FACTORS INFLUENCING THE EFFICACY OF THE IMMUNE RESPONSE TO TRYPANOSOMIASIS

by

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DECLARATION.

This thesis has not been previously submitted for the award of a degree to any university, but is published in part as the following scientific articles:-

(1) P.H. Holmes, J.A. MacAskill, D.D. Whitelaw, F.W. Jennings and G.M. Urquhart (1979)
Immunological Clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice. I. Aspects of the Radiolabelling Technique.
Immunology, 36, 415-419.

(2) J.A. MacAskill, P.H. Holmes, D.D. Whitelaw, I. McConnell, F.W. Jennings and G.M. Urquhart (1980)
Immunological Clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice. II. Mechanisms in Immune Animals.
Immunology, 40, 629-635.

(3) J.A. MacAskill, P.H. Holmes, F.W. Jennings and G.M. Urquhart (1981)
Immunological Clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice. III. Studies in Animals with Acute Infections.
Immunology (in press).

SUMMARY.

A reliable and simple technique for the *in vivo* labelling of trypanosomes with [^{75}Se]-methionine was developed. Between 97% and 99% of the radioactivity was protein-bound in the trypanosome and spontaneous elution *in vitro* was <10% over 4 hrs. The fate of the labelled trypanosomes after i.v. injection into normal and immune CFLP mice was studied. In the latter the liver was found to be the principal site of trypanosome removal.

This finding was further investigated in terms of the respective roles of antibody, macrophage activation and complement in the removal of trypanosomes from the circulation of immune mice. It was found that clearance in such animals was largely accomplished by antibody-dependent hepatic uptake; which at low antibody titres in passively immunised animals was dependent upon C3. In contrast, at high antibody titres in passively or actively immunised mice, the hepatic uptake was independent of complement. No evidence was found to suggest that intra-vascular lysis or activated macrophages were involved in immune clearance.

In studies with ^{75}Se-labelled trypanosome, antibody mediated hepatic uptake could not be demonstrated in mice with acute fulminating *T. brucei* infections. This was not due to impaired macrophage function but was apparently caused by the inability of antibody production to cope with the massive parasitaemias produced by rapidly replicating infections, so that effective opsonisation of the parasites did not occur. In contrast, a strain of trypanosome which causes a more chronic infection, although initially having a similar doubling time, subsequently switched to a slower one, and thereby allowed antibody to reach levels which permitted effective opsonisation.

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There was no evidence to suggest that the parasite caused any significant suppression of antibody responses in these acute infections since inoculation with trypanosomes of one stock at the same time as vaccination with irradiated organisms of a second stock, did not prevent the development of antibody to the latter, as measured by the hepatic uptake of radiolabelled parasites.

Genetic resistance to T. congolense infections or trypanotolerance was investigated using a mouse model in which C57Bl mice were able to repeatedly limit the numbers of circulating parasites, while CFLP mice were unable to do so and died with a fulminating parasitaemia. It was found that infected C57Bl mice were able to remove radiolabelled parasites from their circulation, while CFLP mice could not. The immune response, as measured by in vitro trypanolytic activity and immunoglobulin levels, was found to be better in C57Bl mice. No evidence was found to suggest that immunosuppression was important in the response to trypanosome antigen, as judged by the ability of chronically infected animals to respond to an irradiated trypanosome vaccine, although immunosuppression was readily demonstrable to sheep red blood cells. Furthermore, activation of the mononuclear phagocytic system or passive immunisation did not significantly alter the response of the majority of susceptible animals to infection.

It was concluded that the genetic resistance of C57Bl mice to T. congolense infection was due to the efficacy of their immune response and, in particular, to the ability to maintain high levels of plasma IgM.

Parasite-induced immunosuppression has been suggested to be of importance in both the susceptibility of animals to secondary infection and in the response to vaccination. The latter was investigated in T. congolense/

T. congolense infected cattle vaccinated against Foot and Mouth Disease virus. It was found that the antibody response of infected cattle to vaccination was consistently lower than non-infected animals. This reduced antibody response was directly related to protective antibody levels by challenging cattle with live virus. The results indicated that vaccinated infected animals were more susceptible to viral challenge than vaccinated control animals. Furthermore, chemotherapy at the time of vaccination partially enhanced protection.

GENERAL INTRODUCTION.

Perspective.

The serious threat that trypanosomiasis poses to the economic and social development of sub-Saharan Africa has probably never been greater. The causative agents of this disease which affects both man, his domestic livestock and wild game are protozoan haemoflagellates comprising several species of the genus Trypanosoma. The parasite is in general transmitted cyclically by the haematophagous tsetse fly (Glossina spp.). This disease-vector complex is reported to prevent the rearing of livestock in approximately 10 million square kilometers of tropical Africa, approximately 37% of the continent (Buxton, 1955; Ford, 1975). Furthermore, this area has the potential to support 120 million cattle (Jasiorowski, 1972) and an estimated capital value of U.S. S 5,000,000,000 (Jahnke, 1974). Thus there is a strong economic argument for the eradication of the tsetse and trypanosomiases.

Since their presence has not only deprived these areas of meat but also animal manure to fertilise the soil, milk to supplement the diet and draught animals for cultivation, the disease-vector complex is closely allied to social development and land use in affected areas.

Classification.

The classification of the causative agents of the disease are essentially as described by Hoare (1972) and are outlined in Fig. I.1. The genus is divided into two main sections, the SALIVARIA and the STERCORARIA, depending on whether the parasites complete their development in the salivary glands or in the hind gut of their arthropod vector. Both sections are represented in Africa, although only the salivarian parasites are of medical and veterinary importance as they cause sleeping sickness in humans and nagana in domestic animals.

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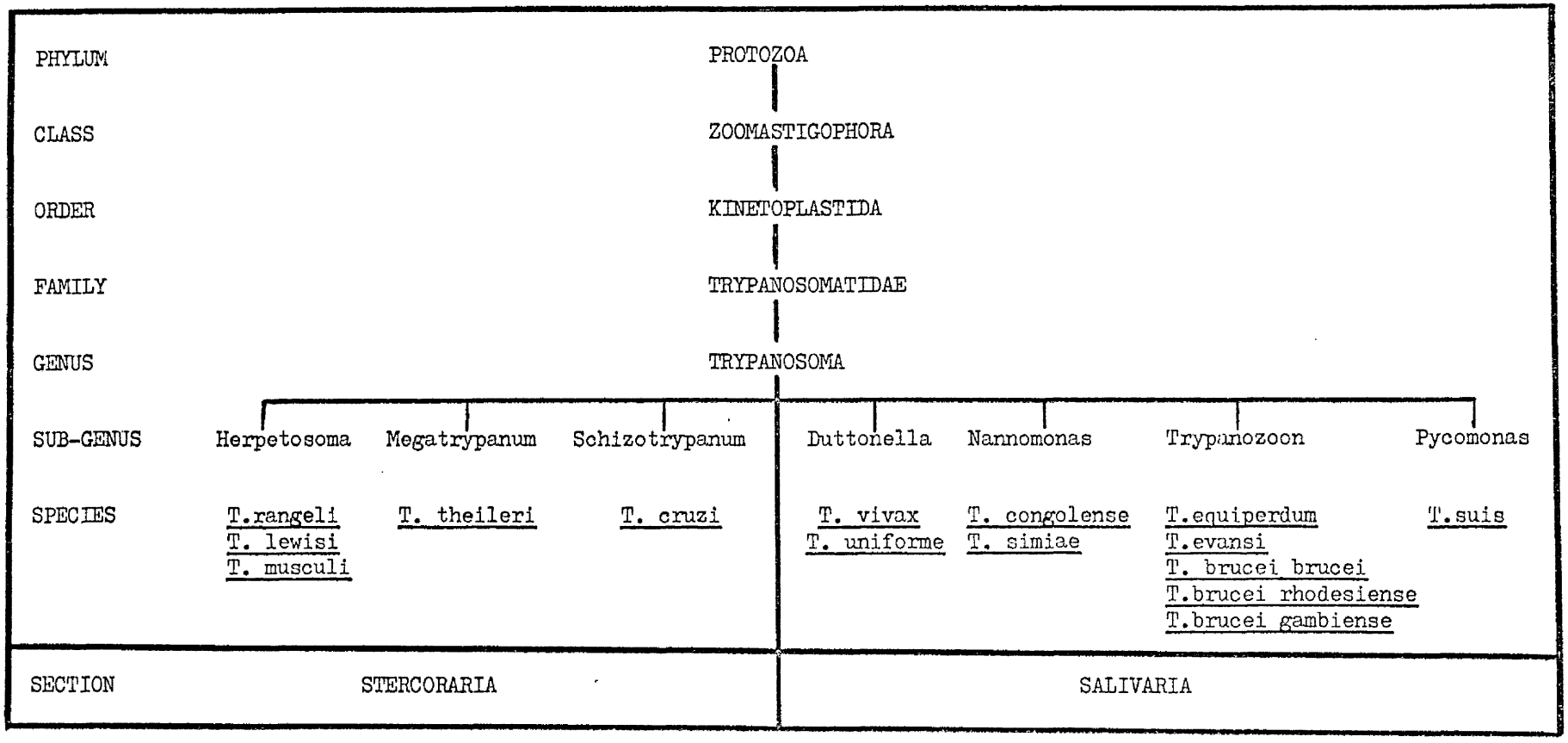
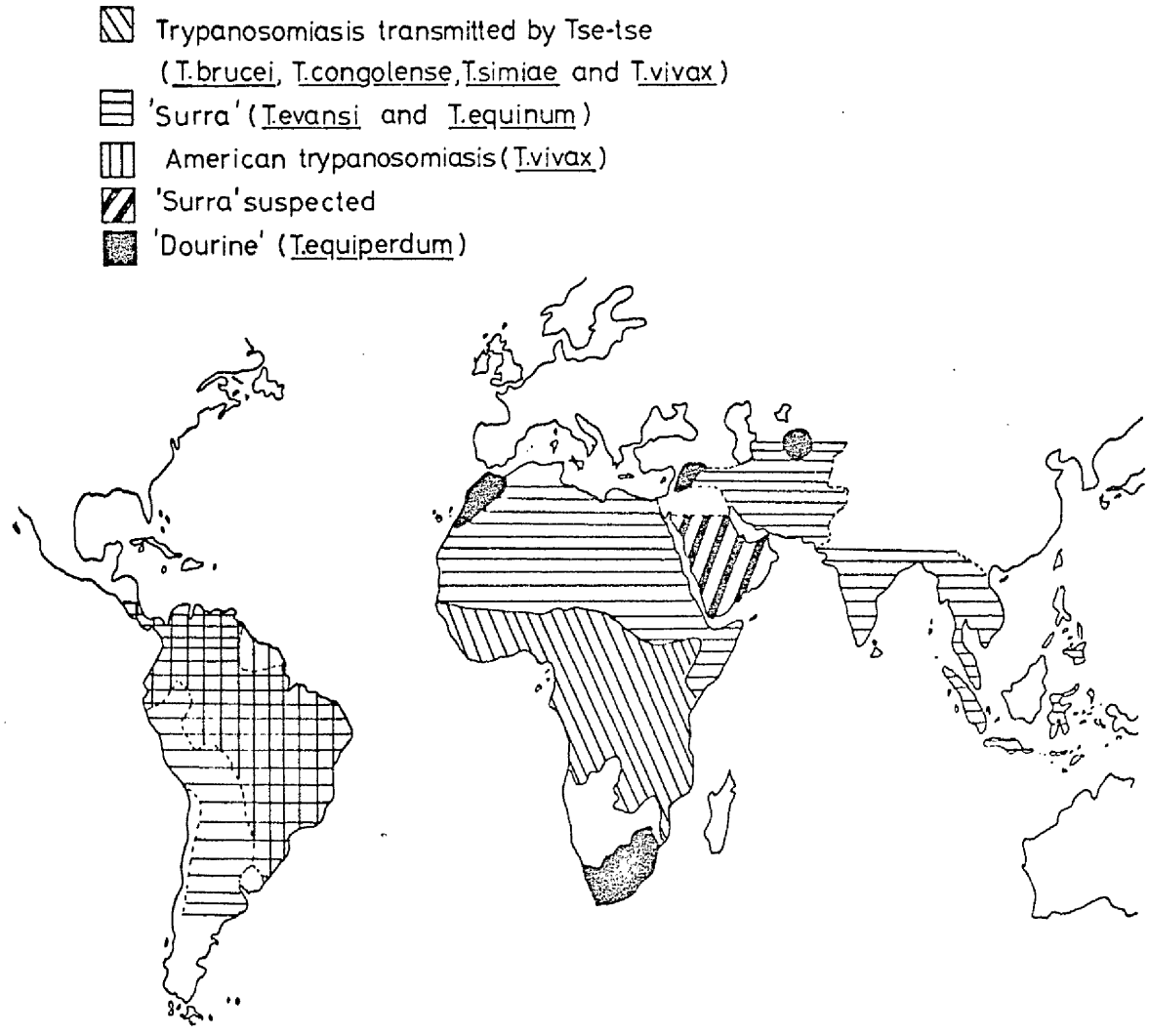
FIGURE I.1 The Classification of Trypanosomes.



After Hoare, C.A. (1972)

Figure I.2 The geographical distribution of the animal trypanosomiases



World Animal Review (1976)

The major pathogens of cattle are Trypanosoma (Nannomonas) congolense and T. (Duttonella) vivax, while T. (Trypanozoon) brucei has until recently been considered to be only mildly pathogenic.

African trypanosomiasis occurs widely throughout the continent except in the arid north and extreme south, affecting cattle, sheep, goats, horses and dogs to a greater or lesser extent. Although this thesis is concerned with African trypanosomiasis, it should also be realised that trypanosomiasis is an important disease outwith tropical Africa (Fig. I.2).

As stated earlier, no major disease is associated with Stercorian parasites in Africa, although T.(Megatrypanum) theileri infections are frequent but usually asymptomatic and undetectable. However, Stercorian parasites do cause serious problems elsewhere and especially in South America where American human trypanosomiasis is the result of T. (Schizotrypanum) cruzi infection. This parasite is transmitted in the faeces of reduviid bugs and seriously affects many millions of people.

Current methods used in the control of African bovine trypanosomiasis.

Tsetse and trypanosomiasis control requires not only careful planning of eradication campaigns but controlled and organised resettlement of the cleared land. This is required if the land, so expensively cleared of tsetse, is not to be squandered by overgrazing and eventual soil erosion. In contrast, if settlement does not take place quickly then re-invasion by tsetse will rapidly occur. Geographical isolation is rarely possible, thus cleared land will frequently abut uncleared land. This necessitates not only constant monitoring of tsetse control campaigns but also veterinary supervision of cattle to control the disease. Unfortunately, surveillance is usually minimal in such areas.

Trypanosomiasis control has historically been divided into two main strategies directed either against the vector or the parasite itself.

Vector control has in general been carried out either by spraying residual insecticides or by bush clearing. Ground spraying of insecticides on resting sites, favoured by tsetse after its blood meal, can be a very successful method of control, although the precedure is manpower intensive and more recently aerial spraying has become the preferred method of dispersal (Burnett, 1970). However, controversy over residual insecticides has meant that alternative chemicals which are less persistent are now more commonly used. As a consequence, more frequent spraying campaigns are required and this has escalated the already high cost.

Bush clearing (Ford, Nash and Welch, 1970) provides a rapid decrease in the breeding sites available for the tsetse. This method can also be used to form a corridor to separate land cleared of tsetse from uncleared areas, provided livestock movement across these corridors is strictly controlled. The culling of wild game has also been occasionally used (Ford, 1970) as these animals were considered to be an important reservoir of the disease. However, this policy is now generally considered to be unacceptable.

In recent years several interesting alternatives have been employed including the use of the sterile male technique (Dame, 1970). One limitation of this method of control is that there is rarely a defined boundary to prevent sterile males leaving or non-sterile males entering the control area. Furthermore, a considerable amount of the available resources have to be diverted into the maintenance of boundaries by insecticide spraying.

Vector control does not necessarily produce total freedom from the disease as mechanical transmission is known to occur, although its importance/

importance in the epidemiology of human and animal trypanosomiasis is unknown. It is, however, certain that in the absence of tsetse the disease would still flourish. Indeed, the continued mechanical passage in many cases can lead to an increasing virulence (Mesnil, 1912), and in pleomorphic strains there is also a tendency for them to become monomorphic (Oehler, 1914). This is exemplified by T. evansi which presumably originated from T. brucei (Hoare, 1972) but is now totally monomorphic and extremely virulent as a result of its extensive mechanical transmission by haematophagous Diptera.

Therefore in conjunction with vector control, it is necessary to control the bloodstream parasite and this has mainly been achieved by chemotherapy or chemoprophylaxis.

The discovery at the beginning of this century that Trypan red was both curative and prophylactic in experimental trypanosomiasis led to a rapid expansion both in trypanocidal drug research and in its chemotherapeutic use in the field. The majority of these early drugs were as a by-product of the German dye-industry, and their potential in human sleeping sickness was quickly recognised. Further advances led to the development of Atoxyl, an organic arsenical derived as a by-product of triphenylmethane dye industry, which was shown to be trypanocidal and non-toxic to humans. The development of these aromatic arsenicals led to an expansion in the therapeutic use of arsenic culminating in the development of Tryparsamide (Williamson, 1970).

In 1920 trials using Tryparsamide were commenced in a campaign against human sleeping sickness in the Belgian Congo. By 1925 it was reported that the drug was highly effective (Williamson, 1970). The successful treatment of a French medical officer with Atoxyl and intravenous tartar emetic led Bevan (1928) to try these new drugs against animal trypanosomiasis, with great success.

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The use of chemotherapy and chemoprophylaxis has grown continually until they now form an important line of defence against trypanosomiasis. The major drugs in use at the present time are Berenil (Diminazene Aceturate, Fabwerkehoechst) as the main curative drug and Samorin (Isometamidium Chloride, May & Baker) as the major prophylactic drug, although it has some curative properties. The principal problem associated with long term chemotherapy is the generation of drug resistant parasites (Whiteside, 1963; Maclennan and Jones-Davies, 1967). Although drug resistance has arisen, it can be minimised by the alternate use of different drugs. This is the situation in many countries at the present time with cattle maintained under chemo-prophylaxis being given occasional doses of curative drugs to reduce the appearance of drug resistance. However, Berenil has been the only new trypanocide to be made commercially available since the late 1950s and whether it would be possible to quickly produce a new drug should the present drugs become ineffective is unknown. The demand for these drugs is limited and the potential profit too low to merit expensive research. Thus there is a pressing need to re-evaluate the dependence of trypanosomiasis control on pharmaceuticals.

Immunity to African trypanosomes.

The archetypal trypanosome infection comprises a series of rising parasitaemias between which are period of remission when few, if any, parasites can be detected in the peripheral circulation. Each new parasitaemia is produced by a variant trypanosome population with a slightly altered surface antigen.

Such antigenic variation is now considered to be one of the crucially important mechanisms involved in the parasite's escape from the host's immune response. Vickerman (1969) described the existence of a coat surrounding the bloodstream forms of T. brucei which was lost when the parasites were grown in culture or in the mid-gut of the tsetse but was regained by the metacyclic trypanosomes in the salivary glands.

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He therefore suggested that the coat was in some way an adaptation to allow the parasite to survive in the plasma of its host. Weitz (1960) had previously described an exoantigen from the plasma of infected blood. This exoantigen was shown to produce protective antibodies against the infecting parasite from which it was derived (Weitz, 1960; Seed and Weinman, 1963). The secretion of this exoantigen from the surface of the parasite into the plasma in vivo was considered to be one mechanism by which the parasite was able to avoid the antitrypanosome antibody (MacAdam and Herbert, 1970a). Whether secretion of the surface coat does occur during an infection is still in dispute.

The ability to produce an immune response against a trypanosome infection has been regularly demonstrated either by infection following by drug cure (Fulton and Lourie, 1945), vaccination with exoantigen (Herbert and MacAdam, 1971; Duxbury, Sadun, Schoenbechler and Stroupe, 1974) or vaccination with radioattenuated trypanosomes (Dusbury and Sadun, 1969; Duxbury, Sadun and Anderson, 1972; Wellde, Schoenbechler, Diggs, Langbehn and Sadun, 1975). This immunity is, however, only directed against the antigenic variant used for the vaccination (Gray, 1967; Herbert & Lumsden, 1968; Wellde et al, 1975; Terry, 1976). Thus the immune response of the host can successfully eliminate the predominant variant from the circulation. It is, however, unsuccessful in controlling the infection as a new variant repopulates the blood.

The process of antigenic variation is now thought to be the result of selective expression and suppression of genes controlling the formation of the surface coat (Van Meirvenne, Magnus & Vervoort, 1977) rather than by a series of random mutations arising from cellular division (Watkins, 1964). The series of variations occurring during an infection is apparently limitless and there are few reports of the reappearance of old variants, although this perhaps reflects limitations in the serological techniques available. However, a clone of T. equiperdum has recently been shown to generate 101 different variants (Capbern, Giroud, Baltz and Mattern, 1977). One defect in the armoury of the trypanosome during its cyclical/

cyclical transmission in the tsetse has been described. This is the tendency for trypanosomes to resort back to a basic antigenic type (Gray, 1965a, 1975; Cunningham, 1966; Jenni, 1977). Thus during the passage of the trypanosome through the tsetse the ingested antigenic type is displaced by a predominant antigenic type which is then transmitted and, although LeRay, Barry, Easton and Vickerman (1977) have recently shown that clone-derived metacyclics display a variety of antigenic types, there may be a predominant variant capable of initiating an infection similar to the original one (Van Meirvenne et al, 1977).

Thus the process of antigenic variation enables the parasite to remain one step ahead of the host's immune response and also prevents the development of an effective vaccine.

Although the development of immunity to trypanosomiasis in domestic animals is only occasionally a sequel to natural infection due to the parasites' ability to undergo repeated antigenic variation, protective immunity against a particular antigenic variant can be readily induced by a variety of vaccination procedures (vide supra: also Terry, 1976; Clarkson, 1976a; Murray and Urquhart, 1977).

Such immunity is thought to depend largely on serum antibody. Thus humoral responses to specific antigenic variants can be demonstrated in vitro by a variety of methods including agglutination (Soltys, 1957a; Cunningham and Vickerman, 1962), trypanolysis (Lourie and O'Connor, 1936), infectivity neutralisation (Soltys, 1957) and metabolic inhibition (Desowitz, 1956; Diggs, Flemings, Dillon, Snodgrass, Campbell and Esser, 1976). The important role of the humoral response in the control/

control of the parasitaemia has been further demonstrated by the use of various immunosuppressive regimes. Luckins (1972) and Balber (1972) have shown that x-irradiation of the host prior to infection prevents a successful immune response and a fulminating infection results. Similarly using immunosuppressive drugs Ashcroft (1957) and Luckins (1972) have shown that failure to produce detectable antibody results in the host's inability to control the infection. While these results are indicative of a failure in the humoral response, the methods used to suppress the immune response in these studies may not only interfere with the B cell response but probably affected the co-operation between various cell populations which is known to regulate the immune response (Allison, Denham and Barnes, 1971). However, subsequent experiments using cyclophosphamide at doses known to suppress B cell responses (Turk and Poulter, 1972; Kolb, Poupon, Lespinats, Sabolovic and Loisillier, 1977) have confirmed these earlier findings (Vickerman, Sless, Haston and Edwards, 1977). Furthermore, the success of passive immunisation using immune serum to confer protection strongly supports the importance of the humoral response (Seed and Gam, 1966; Patton, 1972; Takayanagi, Kambara and Enriquez, 1973).

Transfer of immune spleen cells can also produce strong immunity (Takayanagi et al, 1973a; Takayanagi and Nakatake, 1975; Campbell and Phillips, 1976). In addition, suppression of B cells by anti μ-antibody administration from birth has been shown to prevent an effective immune response. Thus it was found that anti μ treated mice had a decreased survival time compared to control mice and failed to respond to an irradiated trypanosome vaccine (Campbell, Esser and Weinbaum, 1977). An intact B cell response would therefore appear to be essential for the development of an immune response to trypanosome infection.

The effectiveness of this humoral response in controlling a trypanosome infection is apparently dependent on the availability of IgM, IgG and complement.

There is a well documented rise in serum IgM during infection (Mattern, 1964; Lumsden, 1965; Kobayshi and Tizard, 1976; Clarkson, 1976). The continually changing antigenic stimulus during trypanosome infection presumably stimulates only the primary immune response, i.e. predominantly IgM (Seed, Cornille, Risby and Gam, 1969). This means that the immune response is essentially restricted to the circulation, as the large IgM molecule is slow to come to equilibrium with tissue fluids (Goodwin and Guy, 1973; Terry, 1976). Thus parasites circulating in blood vessels will be removed, although parasites which occupy tissue sites such as T. brucei may escape the trypanocidal effects of the circulating antibody (Seed and Effron, 1973). Furthermore, Takayanagi and Enriques (1973) have suggested that IgM is more efficient than IgG in protecting the host from infection. IgM is also recognised to be a better activator of the complement system than IgG. Trypanolysis may therefore be one possible mechanism by which parasites can be removed from the circulation. Trypanolysis occurs readily in the presence of guinea pig serum (Lourie and O'Connor, 1936). They, however, recorded in their introduction the failure of immune mouse serum alone to initiate trypanolysis, presumably as a result of a deficiency in the complement system. So clearly caution must be exercised when discussing the importance of trypanolysis in experimental and natural trypanosomiasis.

Many authors have described the activation of complement during infection (Nagle, Ward, Lindsley, Sadun, Johnson, Berkaw & Hildebrandt, 1974; Jarvinen & Dalmasso, 1976; Nielsen & Sheppard, 1976; Musoke & Barbet, 1977). Fiennes, Jones and Laws (1946) have also recorded hypocomplementaemia as a feature of the disease. If an intact complement system is essential for parasite removal, then hypocomplementaemia induced/

induced by the parasite may be an important mechanism by which the parasite can avoid trypanolysis (Nielsen & Sheppard, 1976; Musoke & Barbet, 1977).

Alternatively the humoral response may act in such a way that it does not directly destroy the parasite but enables its destruction to be initiated. Opsonisation of the parasite by antibody can occur in the absence or presence of complement, although C3 has an important opsonic role in many reactions (Pepys, 1976).

Opsonisation, however, requires additional cellular involvement, usually in the form of phagocytic cells. The immune adherence reaction of antigen to cells was demonstrated early this century (Nelson, 1953; Nelson, 1963) when it was shown that trypanosomes incubated in immune serum attached to platelets and monocytes.

The phagocytosis of trypanosomes by macrophages clearly must involve some method of attachment of the parasite to the cell surface. The demonstration of both C3 and Fc receptors on the surface of macrophages may facilitate this attachment (Berken & Benacerraf, 1966; Mantovani, Rabinovitch & Nussenzweig, 1972; Bianco, Griffin & Silverstein, 1975; Hopf, Meyer zum Buschenfelde & Dierich, 1976). Antibody need not be attached to the target cell but may be present on the surface of the effector cell. Thus cytophilic antibodies (Boyden, 1963; Berken & Benacerraf, 1968) may be opsonic antibodies which act via attachment to the effector rather than the target cells, although Tizard (1969) has described both cytophilic and opsonic antibodies in mice which appear to be distinct. The temperature at which cytophilic antibodies can be detected in mice is usually lower than normal body temperature so their biological importance is uncertain (Tizard, 1969). Agglutination of the parasite is known to be mediated via $F(ab')_2$ fragment of IgG (Takayanagi & Nakatake, 1977), while the attachment to macrophages occurs via the Fc region of IgG (Takayanagi & Nakatake, 1977). It is therefore possible that/

that agglutination plays a role in phagocytosis or attachment to phagocytes and thereby confers protection. Consequently, the agglutination titre may be a useful indicator of the protective power of the serum (Takayanagi, Nakatake & Enriques, 1974).

The important relationship between the humoral response and the mononuclear phagocytic system (MPS) is well established and it has been known for many years that immune serum influences the phagocytic ability of macrophages (Laveran & Mesnil, 1901). These workers observed that T. lewisi were phagocytosed in the peritoneal cavity of rats which had been actively or passively immunised. This was subsequently confirmed in vitro (Lange & Lysenko, 1960; Patton, 1972). A similar relationship has also been demonstrated in African trypanosomiasis (Lumsden & Herbert, 1967) where peritoneal exudate cells exhibited better phagocytic ability against T. brucei in the presence of immune serum. This has since been confirmed on several occasions (Cook, 1975; Takayanagi et al, 1974).

The dependency of phagocytosis on antibody was recently confirmed by Takayanagi, Nakatake & Kato (1977) who also suggested that optimal phagocytosis occurred in a situation of slight antibody excess. Furthermore, this attachment has been shown to be independent of complement (Takayanagi et al, 1974a). Hypocomplementaemia (vide supra) therefore may not be an importment impediment to attachment and phagocytosis, at least in antibody excess.

Despite these studies, it is not known whether whole opsonised trypanosomes (Lange & Lysenko, 1960) or particulate debris resulting from intravascular lysis (MacNeal, 1904; Talliaferro, 1924) are phagocytosed.

A further possibility is that non-specific phagocytosis of trypanosomes may contribute to the protective immune response. Several authors have described the successful use of non-specific MPS modulators

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to enhance phagocytosis of carbon and bacteria (Stiffel, Mouton and Biozzi, 1970). Furthermore, activation of macrophages has been reported to increase protection against Plasmodium sp. and Babesia sp. (Clark, Allison & Cox, 1976; Clark, Cox & Allison, 1977). Activation of macrophages may also be achieved in animals by products of activated T lymphocytes, immune complexes and complement cleavage product C3b (Allison & Clark, 1977). Since all three are readily detectable in trypanosome infected animals, increased hepatic uptake of SRBC in such animals (Murray, Jennings, Murray & Urquhart, 1974) could well be a result of macrophage activation.

One notable feature of trypanosome infections is hypertrophy and hyperplasia of lymphoid and hepatic tissue (Ormerod, 1970; Fiennes, 1970; Murray et al, 1974a,b; Murray, Murray, Jennings, Fisher & Urquhart, 1974). Since it is known that the immune response is dependent upon cellular interaction and co-operation between T lymphocytes, macrophages and B lymphocytes (Allison et al, 1971), the cellular expansion observed during trypanosome infection may result in alterations to the efficacy of the immune response.

Many such dysfunctions have been described in infected animals. The levels of serum IgM are characteristically elevated during trypanosomiasis (Clarkson, 1976a; Hudson, Byner, Freeman and Terry, 1976), although only part of this response (5%) is thought to be specific for trypanosomes (Houba, Brown & Allison, 1969; Freeman, Smithers, Targett & Walker, 1970). The remaining antibody consists of various heterophile responses and autoimmune antibodies (Houba, Brown & Allison, 1969; Freeman et al, 1970). In addition, a decrease in agglutinin response to SRBC during an infection (Goodwin, Green, Guy and Voller, 1972) and a reduction in the plaque forming cell response (PFC) to SRBC (Murray et al, 1973; Hudson et al, 1976) has been reported. Although there was a reduction in the response to vaccination with SRBC during infection/

infection, the background PFC response increased (Longstaffe, Freeman & Hudson, 1973; Hudson et al, 1976), as did the production of heterophile antibody (Houba, Brown & Allison, 1969). This observation, together with the finding of Dresser (1972) that IgG production is almost totally T cell dependent, while IgM production is only partly so, suggests a breakdown between T lymphocyte and B lymphocyte co-operation in infected animals (Terry, Freeman, Hudson & Longstaffe, 1973). This profound immunosuppression was found to occur within a few days of infection (Longstaffe et al, 1973; Murray et al,1974a,b)and resulted in a reduction in specific antigen IgM responses to between 5% and 20% of normal and an almost total suppression of specific IgG responses (Freeman, Hudson & Byner, 1974).

Two mechanisms have been proposed to account for the immunosuppression that results after infection. First antigenic competition and secondly polyclonal B cell activation.

Terry (1977) summarised antigenic competition as being mediated via T cells and macrophages rather than directly at the B cell level. Two mechanisms have been proposed. First, competition resulting from the production of a DNA inhibiting glycoprotein from T cells following antigenic stimulation (Waksman, 1977). This factor results in non-specific inhibition of the target cell's DNA synthesis. Secondly, competition may occur at the macrophage level (Taussig & Lachmann, 1972; Taussig, 1972; Feldman & Nossal, 1972; Feldman, 1976). In this form T cells following antigenic stimulus produce a "co-operating antibody" IgT. IgT or IgT/antigen complex with specific antigen activity then binds to Fc receptors on a macrophage. Attachment thereby allows the correct/

correct presentation of antigen to B cells. If another antigen is introduced during the response to the first antigen, then there will be a reduced number of Fc receptors to bind the second IgT. Fewer antigen particles will then be available for proper presentation to B cells and a diminished antibody response will result.

During an infection the host is presented with a considerable array of antigens, almost simultaneously. If co-operation between T lymphocytes and macrophages is required to produce an effective immune response to these antigens, then antigenic competition can be readily considered to be a mechanism for parasite induced immunosuppression. However, antigenic competition does not explain T cell independent antigen immunosuppression (Murray, Jennings, Murray & Urquhart, 1974a; Eardley & Jayawardena, 1977; Corsini, Clayton, Askonas & Ogilvie, 1977).

Alternatively, immunosuppression may result from polyclonal B cell activation leading eventually to B cell exhaustion. This hypothesis correlates well with findings both *in vitro* and *in vivo*. First, there is a large increase in serum IgM during trypanosome infection which is largely non-specific (Mattern, Masseyeff, Michel & Peretti, 1961; Houba, Brown & Allison, 1969). Secondly, when plaque forming cell assays (PFC) are carried out during trypanosome infections an increase in the background PFC of non-immunised infected animals is found (Freeman, Hudson, Longstaffe & Terry, 1973; Hudson *et al*, 1976). Thirdly, it has been shown that while spleen cells obtained early in the infection are capable of enhanced immunoglobulin synthesis *in vitro*, as the infection progresses this ability rapidly declines even in the presence of a known B cell mitogen (Corsini *et al*, 1977). These findings suggest that polyclonal B cell activation occurs during infection and eventually results in a reduced ability of B cells to produce antibody.

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The observation that polyclonal activation occurs during infection has prompted the suggestion that the parasite produces a mitogen which promotes B cell activation (Urquhart, Murray, Murray, Jennings & Bate, 1973; Greenwood, 1974; Esuoroso, 1976) although Mansfield, Craig and Stelzer (1976) were unable to demonstrate such a mitogenic effect in trypanosome extracts.

Two other factors which may contribute to immunosuppression are hypocomplementaemia and the release of polyunsaturated fatty acids from lysed parasites. Complement, and especially C3, is known to be required in the induction of antibody synthesis (Pepys, 1976) by initiating attachment of antigen complexes to dendritic cells in the spleen and in the formation of B memory cells (Klaus & Humphrey, 1977). The depletion of C3 may therefore result in a reduction in the trapping of antigen in the spleen and lead to a diminished immune response.

Autolysis of parasites has been shown to release immunosuppressive polyunsaturated fatty acids (PUFA)(Tizard, Nielsen, Mellors & Assoku, 1977; Assoku, Tizard & Nielsen, 1977). This mechanism may exert its most important effect as a reaction at the site of trypanolysis and may thus alter local lymphocyte function, rather than having a generalised effect.

The key question in parasite induced immunosuppression is whether or not the immunosuppression affects the host's response to the parasite. Hudson et al (1976) correlated the failure of infected mice to control a second parasitaemic crisis with the immunosuppression induced by the first parasitaemic crisis. However, the variety in pattern and course of trypanosome infections, although associated with a profound degree of generalised immunosuppression, at least to T dependent antigens, would seem to suggest that the full significance of immunosuppression in trypanosomiasis remains to be determined.

Evidence of protective immunity in field situations.

There is at present a growing interest in the reduced susceptibility to trypanosome infection shown by some breeds of cattle and also some smaller ruminants (Stewart, 1951; Toure, 1977). This ability to limit the severity of the disease has been termed trypanotolerance.

The basis of trypanotolerance is unknown, although it has usually been considered to be an inheritable trait, i.e. a genetic resistance. However, it is important to distinguish this form of genetic resistance from forms of selective species resistance such as that shown by man to T. brucei infection (Hawking, 1973) and by the cotton rat to infection with a rodent adapted T. vivax (Terry, 1957).

In general the indigenous breeds of West African cattle are considered to display the greatest degree of trypanotolerance, while the comparatively recently introduced Zebu and other exotic breeds are usually highly susceptible to the effects of trypanosomiasis (Chandler, 1952, 1958; Desowitz, 1959; Stephen, 1966; Roberts and Gray, 1973a; Murray, Murray, Wallace, Morrison & McIntyre, 1977; Toure, 1977).

There are only a few experiments in cattle which have investigated the genetic basis for the resistance of N'dama cattle. Stewart (1951) reported that cross breeding between West African trypanotolerant breeds and susceptible Zebu cattle resulted in offspring which showed a marked degree of trypanotolerance. These differences may well be due to the large degree of outbreeding in cattle resulting in an uneven distribution of the genes involved in conferring trypanotolerance. Indeed, it may be expected that the degree of trypanotolerance, at least in first generation offspring may be directly related to the parental gene combination (Roberts and Gray, 1973a).

Desowitz/

Desowitz (1959) showed that the immune response of N'dama cattle was superior to that of Zebu cattle only if the N'dama had been reared in a tsetse infested area. If the offspring of trypanotolerant N'dama cattle were born and bred in a tsetse-free area, they were as susceptible as Zebu cattle to infection, as were the normally trypanotolerant Muturu cattle which had been maintained for several generations in a tsetse-free area. These results suggest that there may be an important aspect of trypanotolerance which is acquired.

However, more recent studies (Murray et al, 1977) using N'dama and Zebu cattle obtained from areas considered to be free from trypanosomiasis have indicated that genetic factors may be crucial in determining the susceptibility of cattle to trypanosomiasis. Thus, following experimental infection with T. congolense, N'dama cattle had lower parasitaemias; developed a milder anaemia; lower mortality and fewer pathological lesions than Zebu cattle given the same infection.

In addition to the genetic resistance described above, there is considerable field evidence to suggest that significant levels of acquired resistance can develop in normally susceptible cattle if they are given strategic chemotherapy. This phenomenon was first described by Bevan (1928) and by several workers subsequently.

Fiennes (1970) described the experience of a Kenyan farmer who maintained Zebu cattle in an area of tsetse challenge under routine chemotherapy. Gradually over the years it was noted that the intervals between drug therapy were increasing and the number of abortions were decreasing. It was especially interesting that, although the calves became infected at an early age, the incidence of adult trypanosomiasis

was/

was low. Similarly, Wilson, LeRoux, Paris, Davidson and Gray (1975) reported a series of experiments designed to investigate acquired immunity. They found that cattle maintained in a high tsetse challenge area, treated on an individual basis either when clinically ill or when their PCV fell below 0.20, produced an increase in live births and a decrease in the number of abortions. There was, however, no development of protective immunity as continual regular drug therapy was required. In contrast, when cattle were maintained in an area of medium tsetse challenge (Wilson, Paris, Luckins, Dar and Gray, 1976), the interval between drug treatments increased while control cattle introduced at regular intervals became infected and required regular chemotherapy.

Thus, while the development of acquired immunity is practical in susceptible animals, it is dependent upon the level of tsetse challenge and on careful chemotherapy with a high degree of veterinary supervision.

Genetically resistant animals clearly have considerable potential in many areas of Africa, yet the basis of their resistance remains unknown. Unfortunately, studies of genetic resistance in cattle are restricted to Africa and are therefore difficult and expensive to organise. Studies have therefore turned to mice in an attempt to investigate genetic resistance to infection. Ssenyonga (1974) reported that C57Bl mice survived a T. congolense infection for a much longer period than CBA mice. This work has been studied further by Jennings and Whitelaw (1977) using a variety of mice strains, and a variety of T. congolense stocks. These workers found that C57Bl mice showed a consistently higher degree of trypanotolerance as judged by their extended survival following infection. This resistance did not require previous exposure to infection and was therefore truly an innate trypanotolerance.

The/

The availability of such mice offers an inexpensive and convenient model for the study of trypanotolerance and avoids the problems associated with small group numbers and outbreeding in cattle because of the known genetic background of the particular mouse strain used.

This thesis is concerned with an investigation into factors affecting the efficacy of the immune response in trypanosomiasis.

To aid this investigation and enable a direct evaluation of the efficiency of the immune response in removing circulating trypanosomes, a new method for labelling trypanosomes with the amino acid analogue ^{75}Seleno-methionine was developed (Chapter 1).

Secondly, these radiolabelled trypanosomes were used to investigate the mechanisms involved in the removal of trypanosomes by immunised mice and investigates the role and relative importance of antibody, complement and the mononuclear phagocytic system in this removal (Chapter 2).

Thirdly, radiolabelled trypanosomes were used in conjunction with a variety of other techniques to assess the importance of an efficient humoral response in actively infected mice and the dependency on the degree of parasite induced immunosuppression and parasite replication rate on the success of this response in controlling the initial parasitaemia (Chapter 3).

The last two chapters deal with T. congolense infections in mice and cattle respectively.

Chapter 4 investigates the genetic resistance to T. congolense infection and considers whether trypanotolerance is due to differences in the effectiveness of the immune response.

Finally/

Finally, parasite-induced immunosuppression has been suggested to be of practical importance in the response of animals to vaccination. Chapter 5 outlines an investigation into the response of T. congolense infected cattle to Foot and Mouth disease vaccination. The response to vaccination was measured not only by serological assays for antibody but also in the ability to resist live virus challenge.

GENERAL MATERIALS AND METHODS.

Experimental Animals.

Mice:

These were:-

(a) Randomly outbred female CFLP mice originally obtained from Anglia Laboratory Animals (Huntingdon) and subsequently from Hacking and Churchill Ltd. (Huntingdon). Both colonies of mice were originally derived from the I.C.I. strain 1 mice (I.C.I., Alderley Park, Cheshire). The mice weighed between 25g. and 30g. and were 6-8 weeks of age at the beginning of each experiment.

(b) Inbred female, C57Bl mice obtained from the Laboratory Animal Centre (Edinburgh). The mice weighed between 20-25g. and were approximately 8 weeks of age at the start of each experiment.

Rats:

These were random bred female Hooded Lister rats obtained from OLAC 1976 Ltd. (Bicester) weighing between 160g. and 180g. and were approximately 10 weeks of age at the beginning of each experiment.

Feeding and Housing:

All animals were housed in metal/plastic cages with sawdust bedding changed twice weekly. The cages were held in metal stands in an animal house which had a temperature of 20°C. ± 2°C. The animals were fed ad libitum on diet 41 (Angus Milling Co., Perth). Drinking water was constantly available to all mice from glass or plastic bottles. Antibiotics were not administered.

Infection:

Animals were infected with a known number of trypanosomes obtained from stabilates stored in liquid nitrogen. Immediately prior to infection a portion of stabilate tubing was re-suspended in ice cold phosphate/

phosphate buffered saline (PBS) containing 15g. glucose per litre (PBGS) pH 8.0. Mice and rats were infected with 1 x 10^4 and 1 x 10^5 organisms respectively by intraperitoneal (ip) injection.

Trypanosomes.

Origin of stabilates:

The trypanosomes used in these experiments can be divided into two subgenera:-

(i) Trypanozoon: These were kindly supplied by Dr. A.R. Gray from The Centre for Tropical Veterinary Medicine, Edinburgh. The original isolates were numbered Trypanosome Research Edinburgh University (TREU) 226 and 667, and both were described as Trypanosoma brucei brucei. TREU 226 was originally isolated from an experimentally infected cow in June, 1961, at the East African Trypanosome Research Organisation as EATRO 38

EATRO 38	
↓	3 days mice
TREU 16	
↓	3, 3, 3 days
TREU 55	
↓	2, 3, 3, 2, 2 days
TREU 62	
↓	3, 3, 3, 2, 2, 3, 2 days
TREU 70	
↓	3, 2, 2, 3, 2, 2, 2 days
TREU 87	
↓	3 days
TREU 164	
↓	3 days
TREU 226	

This/

This parasite causes a rapidly fulminating parasitaemia with death occurring within 5-6 days.

TREU 667 was originally isolated from a naturally infected bovine host by a University of Edinburgh expedition to Uganda in 1966. The primary isolate number was Busoga/66/EVE/10. The history is as follows:-

TREU 628 Busoga/66/EVE/10.
↓ 22 days
TREU 667

This parasite produces a sub-acute infection in mice lasting 6-12 weeks and in rats 5-8 weeks.

(ii) Nannomonas: This parasite was kindly supplied by Professor K. Vickerman as Glasgow University Protozoology (G.U.P.) 92. The origin of the stabilate is unknown and the parasite was described on the basis of morphology as T. congolense. This parasite produces two distinct patterns of infection depending on the host animal. The infection is acute and fulminating in CFLP mice with the majority of deaths occurring between days 10 and 12, while in C57Bl mice a chronic infection is produced, lasting beyond 100 days, associated with periods of remission and relapsing parasitaemia.

Cryopreservation.

The passage of trypanosomes through animals may eventually result in changes occurring in the antigenic profile of the parasites. To ensure antigenic stability the parasites were stored in liquid nitrogen using glycerol as the preservative at a final concentration of 10% (v/v).

At the beginning of these investigations a reserve stabilate was established for each species of trypanosome.

Lethally irradiated mice with fulminating infections were exsanguinated by cardiac puncture under deep trichloroethylene (Trilene, I.C.I./

I.C.I., England) anaesthesia, using heparin (Heparin (Mucous) Sodium, Evans Medical Ltd., Liverpool) as the anti-coagulant. The blood was transferred to a glass tube and the appropriate volume of glycerol added. The blood and glycerol were gently mixed by repeated inversion of the glass tube and allowed to stand for 1 minute to permit the glycerol to penetrate the trypanosomes. The blood was then drawn into a length of 2 mm. silicon tubing (Portex Ltd., Kent), according to the method of Taylor (1972). The tubing was wrapped around a suitable length of 6 mm. dowelling and the stabilate frozen in the gas phase of liquid N_2 overnight prior to its complete immersion in liquid Nitrogen (-196°C.).

Separation of bloodstream trypanosomes.

Mice with fulminating infections were exsanguinated by cardiac puncture under deep trichloroethylene anaesthesia. The trypanosomes were separated from erythrocytes by DEAE cellulose chromatography (DE52, Whatman Ltd., Kent) according to the method of Lanham and Godfrey (1970). Pre-swollen DEAE cellulose (diethylaminoethyl cellulose) was equilibrated in PBS and the pH adjusted to pH 8.0 with orthophosphoric acid. A glass funnel was then filled with the resulting slurry and ice cold PBGS, pH 8.0 used to bed down the cellulose. The infected blood was layered on top of the cellulose bed and allowed to absorb on to the column. The trypanosomes were washed free from the column with ice cold PBGS, collected and concentrated in a temperature controlled centrifuge (Mistral 4L, MSE, England) at 2000 r.p.m. (650g.) for 20 mins. at 4°C. The resulting trypanosome pellet was washed three times in ice cold PBGS.

The final number of trypanosomes/ml. was determined using an improved Neubauer haemocytometer (Hawksley & Sons Ltd., London), as described by Lumsden, Herbert and McNeillage (1973).

Enumeration./

Enumeration of the parasite.

Two basic methods were used to determine the number of trypanosomes per ml.:-

(a) Neubauer Haemocytometer. This method was used on all occasions apart from the monitoring of bloodstream parasitaemia. The method used was essentially that described by Lumsden et al (1973).

(b) To estimate the number of parasites circulating during an active infection, the "rapid matching" method of Herbert and Lumsden (1976) was used. This method enables the number of circulating parasites to be estimated from a wet smear of tail blood on a $\log_{10}$ scale. The same microscope was used for each routine estimation of parasitaemia. This microscope had been previously calibrated by making serial doubling dilutions of a known number of washed separated trypanosomes in normal mouse blood.

Immunisation.

Mice or rats were immunised by infection followed by trypanocidal drug therapy (Diminazene aceturate, Berenil 40 mg. active principal/kg. body weight, Hoechst, Frankfurt, Germany). The drug was administered in distilled water i.p. These mice were than challenged 4 weeks later with the homologous strain of parasites. Wet tail blood smears were monitored daily for the appearance of parasites. Failure to detect trypanosomes over 4 consecutive weeks following challenge was taken as being evidence of immunity.

Serological Tests.

These were carried out basically as described by Lumsden et al (1973) with the following minor alterations:-

(a) Infectivity neutralisation test. The test was performed in 1 ml. plastic tubes. Each tube contained 200 µl test serum or PBS, 50 µl/

50 µl fresh guinea pig serum (GPS), as a source of complement and 5×10^4 trypanosomes suspended in 250 µl PBGS. After incubation at 4°C. for 30 mins. the contents of each test well were taken up into a syringe and divided equally between 5 mice. These mice were then monitored daily for the appearance of blood parasites. Failure to detect parasites over a 2 week period was taken as evidence for the presence of neutralising antibody.

(b) Trypanosome agglutination test. The test was carried out in microtitre plates (Cooke Laboratory Products, Virginia, U.S.A.). Serial doubling dilutions of the test serum were carried out in PBS. To each test well was added 5×10^7 trypanosomes in 25 µl PBGS. The microtitre plates were incubated at 37°C. for 30 mins. and then read on an inverted microscope (Leitz, Germany) at X 400 magnification.

Preparation of Hyperimmune serum.

Hyperimmune serum (HIS) was obtained from rats infected with T. brucei 226 and subsequent drug cure with Berenil (40mg./kg.) on day 4 of infection; then 4 and 24 days later the rats received two challenges of 1×10^5 organisms i.p. The serum was then collected 9 days later and pooled. This method consistently produced HIS with a trypanosome agglutination titre of at least $^1/64$.

Irradiation of animals.

Animals were sublethally irradiated 1 day prior to their infection with 650 rad in a ^{60}Co source (Nuclear Engineering Ltd., Berks.).

Radiopharmaceuticals.

The isotopes used in these experiments were supplied by the Radiochemical Centre (Amersham). These were [^{75}Se]-methionine (100 µCi/ml)/

(100 μCi/ml) containing 10-50 μg methionine/ml., and carrier free ^{125}I iodide (100 mCi/ml).

Injection of radiolabelled trypanosomes and sampling.

Washed radiolabelled trypanosomes were diluted in PBGS to give a final concentration of 1 x 10^9 trypanosomes per ml. Each mouse then received 0.1 ml. by injection into a tail vein after sedation with 0.03 ml. i.p. of a neuraleptanalgesic mixture comprising of Fentanyl base (0.2 mg./ml.) and Fluanisone (10.0 mg./ml.)(Hypnorm, Janssen Pharmaceuticals, Belgium). After 1 hour, unless otherwise stated, the animals were given an overdose of trichloroethylene and a blood sample taken by cardiac puncture. The spleen and liver were carefully removed. These were then washed in PBGS, dried slightly and weighed. The tissues, together with an aliquot of the blood (0.5 ml.), were placed into individual counting vials. The remainder of the carcase was then divided into three counting vials and the radioactivity of these, together with the spleen, liver and blood sample, determined using an automatic gamma scintillation counter (Packard tri-carb 3330, Illinois, U.S.A.).

The injected activity for each animal was calculated as the sum of the counts per minute (c.p.m.) recorded for each sample. The result for each organ is expressed as a percentage of the total injected activity. The percentage of ^{75}Se-activity remaining in the blood was obtained from the calculated total blood volume of each individual animal. The latter was estimated by multiplying the weight of each animal, expressed in grams, by 0.067 ml. blood per gram of body weight. This factor for blood volume was obtained from earlier studies by application of the dilution principle using ^{51}Cr-labelled red cells.

Statistics.

Variation around the mean is expressed as the standard error.

SECTION I.

IMMUNOLOGICAL CLEARANCE OF ^{75}SE-LABELLED *TRYPANOSOMA BRUCEI* IN MICE.

Chapter 1

ASPECTS OF THE RADIOLABELLING TECHNIQUE

Introduction.

The immune response of animals to pathogenic African trypanosomes has received considerable attention over recent years. The mechanism involved in the removal of the parasite from the circulation is however still unclear (see General Introduction). The development of a technique that could produce viable labelled trypanosomes was seen therefore as an invaluable tool for the investigation of the immune mechanisms involved in trypanosome clearance from the circulation.

This chapter outlines the development of such a technique utilising the incorporation of the amino acid analogue [^{75}Se] -methionine to produce ^{75}Se-labelled Trypanosome brucei.

Several attempts to radiolabel trypanosomes have been reported but so far these methods, using in vitro techniques, have been only partially successful.

The most common method used has been the incorporation of radiolabelled pyrimidines during parasite biosynthesis primarily [^{3}H] (tritiated)-thymidine or [^{3}H] -adenine. It has been shown that these pyrimidines are incorporated into kinetoplast and nuclear DNA (Leninger, 1975; Veins and Targett, 1972; Sanderson, Bunn and Lopez, 1978). For example [^{3}H] -thymidine has been used successfully to label T. mega (Steinert and Steinert, 1962); T. evansi and T. gambiense (Inoki and Tadasuke, 1969); T. brucei (Balber, 1971) and T. musculi (Viens and Targett, 1972), although it is not apparently incorporated into T. vivax (Isoun and Isoun, 1974), presumably due to the parasites' ability to synthesise thymidine de novo.

Tritiated compounds require quantification of the radioactivity by liquid scintillation. This method, although more efficient than measuring gamma ray emissions, requires lengthy sample preparation and the/

the problem of sample quenching can interfere with the interpretation of the results.

The gamma emitting isotope $[^{51}Cr]$-Chromium has been used successfully in cytotoxicity tests (Batchelor, 1973). It was therefore not surprising that attempts be made to label parasitic organisms with this isotope. Butterworth, Sturrock, Houba and Rees (1974) successfully labelled Schistosoma mansoni schistosomula with $[^{51}Cr]$-Chromium. Similarly, Subrahmayan, Rao, Mehta and Nelson (1976) have successfully used ^{51}Cr -labelled Litomosoides carinii microfilaria in a cytotoxicity assay. Unfortunately attempts to label trypanosomes with $[^{51}Cr]$ -Chromium have been less than successful. For example, Kuhn, Vaughn and Ianuzzi (1974) attempted to use sodium $[^{51}Cr]$chromate to label T. cruzi but the labelling efficiency was low even when beta particle emissions were measured by liquid scintillation in preference to gamma emissions.

The use of $[^{99m}]$ Technetium to label T. dionisii(Mkwanazi, Franks and Baker, 1976) was limited by the isotope's short half-life of 6 hrs., and difficulties in its manufacture from $[^{99}Mo]$-molybdenum.

Two major criticims arise when using in vitro cultivation to label trypanosomes. First, the incubation may result in a possible alteration to the parasites surface antigen profile and secondly, the incubation may adversely affect the parasites' subsequent infectivity and replication in the host (Dahlin, Hungerer and Zwischer, 1976).

The problems associated with in vitro incubation may be overcome if the labelling is carried out in vivo, and especially when the isotope is metabolically incorporated into the trypanosome.

In general the readily available ^{3}H pyrimidines have been used. However, in an attempt to overcome their problem of sample preparation for liquid scintillation, yet benefit from the isotopes' metabolic incorporation, a radiolabelled gamma emitting amino-acid analogue was used.

This/

This chapter describes a series of experiments designed to investigate first, the possibility of labelling trypanosomes with [^{75}Se]-methionine, and secondly, the suitability of such radiolabelled parasites for use in immunological clearance studies.

Materials and Methods.

Parasite: The stabilate of Trypanosoma brucei used in these experiments was originally derived from the stock TREU 226.

Experimental Animals: The animals used were female Hooded Lister rats and female CFLP mice.

Radiolabelling Technique: Radiolabelled trypanosomes were prepared in rats with fulminating parasitaemias, usually 5 days after infection, by intravenous (i.v.) injection of 50 μCi [^{75}Se]-methionine. The infected blood was collected 20 hrs. later and the trypanosomes separated from blood cells by DEAE cellulose chromatography (General Materials and Methods).

Injection of Radiolabelled Trypanosomes and Sampling: This was essentially as described in the general Materials and Methods. Briefly, a known number of radiolabelled trypanosomes, usually 1 x 10^8 with a calculated activity, were injected i.v. into each mouse. One hour after the injection of radiolabelled parasites the animals were given an overdose of trichloroethylene and a blood sample collected by cardiac puncture. An aliquot of known volume, usually 0.5 ml., was then dispensed into a counting vial. The abdomen was opened and the spleen and liver were then removed, washed in saline dried on tissue paper and each organ's weight recorded before being placed into separate counting vials. The remainder of the carcase was divided between three counting vials. The tissue distribution of ^{75}Se-labelled trypanosomes was determined by analysis on an automatic gamma scintillation counter and expressed as a percentage of the injected activity (General Materials and Methods).

Immunisation/

Immunisation Procedure: Mice were immunised by infection and trypanocidal drug therapy using diminazene aceturate (Berenil) as described in General Materials and Methods.

Homogenisation and Protein Separation of ^{75}Se-labelled Trypanosomes: A 5 ml. sample of ^{75}Se-labelled trypanosomes containing approximately 5 x 10^8 trypanosomes/ml PBGS was disintegrated by freezing in liquid nitrogen and thawing to 37°C. in a temperature controlled water bath. This procedure was repeated 4 times. The resultant homogenate was then stored at -20°C. until required.

A sample of freshly prepared homogenate was fractionated on a DEAE cellulose column (Lanham and Taylor, 1972) and its protein content and radioactivity quantified. The eluent gradient used was 0.4M phosphate buffer and 0.04M phosphate buffer with 0.8M NaCl added, pH 8.0.

Location of [^{75}Se]-Methionine in Serum Proteins on the Surface of Trypanosomes: (a) Serum albumin and globulin were separated by paper electrophoresis as follows:- a 35 cm length of Whatmans No. 1 filter paper was placed in an electrophoresis tank. The ends of the paper were allowed to dip into 0.09M barbiturate buffer, pH 8.6 and soaked for 1 hr. 0.01 ml of serum was then applied using a wire applicator across the paper, approximately 15 cm from the cathode, leaving about 5 mm either side. The sample was then run for 16 hrs at 100 volts.

The strip was removed, dried and stained in 0.1% bromophenol blue (George T. Gurr, London) dissolved in methanol for 20 mins. It was then washed with dilute acetic acid to remove superficial stain, the final wash was carried out using methanol.

The paper strip was then prepared for radioactivity determination by cutting it into equal segments and placing them into counting vials. The quantities of albumin and globulin were estimated by eluting the protein-bound activity with barbiturate buffer and measuring the protein content spectrophotometrically (Pye Unicam, Sp6-500 U.V. Spectrophotometer, Cambridge).

(b)/

(b) The location of ^{75}Se activity in radiolabelled T. brucei was compared to labelled serum proteins by electrophoresis on cellulose acetate strips (Cellogram, Shandon Southern, Surrey). The procedure was as follows:- cellulose acetate strips were placed on the surface of 0.09M barbiturate buffer, pH 8.6. The buffer was allowed to soak up through the strip, thus preventing air bubbles being trapped in the pores. When thoroughly soaked the strips were immersed in the buffer.

The strips were lightly blotted to remove excess buffer and laid across the supports of an electrophoresis tank. These supports were linked to the buffer by "wicks" made from filter paper. The edges of the cellulose acetate strips were sandwiched between these pieces of filter paper and held in position by perspex rods.

The samples were applied using an 0.03 ml applicator (Shandon Southern, Surrey) 4 cm from the cathode. Each sample was then run for 1 hr. at 150 volts. Following electrophoresis, the strips were dried in a hot oven to fix the proteins, and stained with 2% Ponceus in 20% TCA. Excess stain was removed by washing in 5% acetic acid. The radioactivity determinations were as above. The different plasma protein fractions were estimated on a chromoscan (Joyce-Loebl & Co. Ltd., Gateshead) and expressed as a fraction of the total protein.

Total Protein Estimations: Total protein was measured either by the the Folin phenol method of Lowry, Rosebrough, Farr and Randall (1951) or by measuring the solution at 260nm and 280nm on a spectrophotometer and estimating the protein content from the following equation:-

$$\text{Total protein} = 1.45 \times OD_{280} - 0.74 \times OD_{260} \text{ mg/ml}$$

Protein bound radioactivity of radiolabelled trypanosomes: The protein bound activity was measured after 10% trichloroacetic acid (TCA) precipitation of an homogenised sample. The resultant precipitate was sedimented/

sedimented in a temperature controlled centrifuge at 1500g for 20 mins. at 4°C. The percentage protein-bound radioactivity was calculated as follows:-

$$\frac{\text{c.p.m. of supernate x 100}}{\text{Total counts of pellet plus supernate}}$$

Maintenance of ^{75}Se-labelled trypanosomes in vitro: Freshly separated radiolabelled trypanosomes were maintained in commercial culture medium RPMI 1640 (Gibco-biocult, Paisley)(100 ml RPMI 1640 with 15 mM Hepes and L-glutamine) to which had been added 20 ml foetal calf serum, 1 ml, Molar Hepes buffer (Gibco-biocult, Paisley), 10,000 units Penicillin and 10,000 µg streptomycin (Glaxo Laboratories Ltd., Greenford).

5 ml aliquots of the culture medium were then dispensed into sterile universal bottles and each bottle seeded with 1 x 10^8 trypanosomes. They were then maintained at 37°C.

Results.

Labelling of Trypanosomes with [^{75}Se]-methionine: To investigate the incorporation of [^{75}Se]-methionine into trypanosomes the following two experiments were undertaken:-

(a) Time course of label incorporation:

The labelling efficiency was investigated in an experiment in which two groups of 3 irradiated rats received 50 µCi [^{75}Se]-methionine on the day of infection or 4 days after infection. The trypanosomes were collected 5 days and 20 hrs. later, respectively.

The results presented in Table 1.1 show that the shorter the exposure of the parasite to the isotope the greater the labelling efficiency, i.e. 0.04% after 20 hrs. compared to 0.004% after 5 days.

To investigate the dependence of the specific activity of labelled trypanosomes on the plasma concentration of the isotope, the following experiment was undertaken:-

TABLE 1.1 The relationship between specific activity and labelling time.

Period of incubation	c.p.m. per 10^8 trypanosomes	% labelling efficiency*
20 hrs	4053 ± 259	0.04
5 days	450 ± 89	0.004

*The labelling efficiency was determined from the c.p.m. per 10^8 trypanosomes expressed as a percentage of the total injected activity.

TABLE 1.2 Relationship between specific activity and the injected dose of isotope.

Quantity of isotope	Specific activity per 10^8 trypanosomes
100 µCi	9000 ± 420
50 µCi	5027 ± 301
30 µCi	2359 ± 326

(b) The relationship between specific activity of labelled trypanosomes and injected dose of [^{75}Se] - methionine:

If [^{75}Se]-methionine is incorporated into the parasite at the same rate as naturally occurring methionine, then by raising the quantity of [^{75}Se]-methionine in the circulation, the ratio of [^{75}Se]-methionine to cold (non-labelled) methionine molecules entering the parasite should also rise. This may lead to an increase in the specific activity.

To investigate this a group of 6 irradiated rats with fulminating infections were given an i.v. injection of either 100 μCi, 50 μCi or 30 μCi of [^{75}Se]-methionine. The populations of radiolabelled parasites were then collected 20 hrs. later. The specific activity of these parasites is presented in Table 1.2.

The results show that there is a direct relationship between the injected dose of [^{75}Se]-methionine and the specific activity of the labelled trypanosomes. This would suggest that [^{75}Se]-methionine was readily incorporated into the trypanosomes, probably at the expense of the unlabelled methionine.

Incorporation of [^{75}Se]-methionine into trypanosomes: The uptake of [^{75}Se]-methionine into schistosomal protein (Christensen, 1977) and the rapid appearance of ^{75}Se-labelled host plasma proteins shortly after i.v. injection of [^{75}Se]-methionine into normal rats (Awwad, Potchen, Hoelstein and Dealy, 1966) suggests that it is a metabolic incorporation.

To ensure that the ^{75}Se activity associated with the trypanosomes was the result of [^{75}Se]-methionine incorporation into trypanosome protein rather than the adsorption of labelled host plasma proteins on to the parasites' surface, the distribution of ^{75}Se-activity in host and parasite protein was investigated.

(a)/

(a) Trichloroacetic acid insoluble activity: Separated and washed radiolabelled trypanosomes were homogenised by repeated freezing and thawing. The newly synthesised protein in the resultant supernate was precipitated with 10% Trichloroacetic acid (TCA). The resultant precipitate retained 95-97% of the total radioactivity.

(b) DEAE Cellulose chromatography of trypanosome homogenate: The particulate debris from the trypanosome homogenate prepared by freezing and thawing was removed by high speed centrifugation (1 hr. at 30,000 g). The resultant supernate contained 8.8 mg. protein/ml. A sample of this ^{75}Se-trypanosome supernate was applied to a DEAE cellulose column (1.5 cm. x 25 cm.) and the elute collected at a flow rate of 48 ml./hr.

Figure 1.1 illustrates that the radioactivity of the sample was closely associated with several protein rich fractions.

Location of [^{75}Se]-methionine activity in proteins attached to the surface of trypanosomes:

It has been reported previously that adsorption of plasma proteins on to the surface of trypanosomes may occur (Ketteridge, 1970; Diffley, 1977). Hence the accumulation of ^{75}Se-labelled plasma proteins (Awwad et al, 1966) on to trypanosomes could be a possible mechanism by which the parasites become labelled with ^{75}Se-activity. To investigate this possibility the distribution of radioactivity of an homogenate of ^{75}Se-labelled trypanosomes and ^{75}Se-labelled plasma proteins was compared by paper and cellulose acetate electrophoresis.

The results presented in Table 1.3 show that the majority of the ^{75}Se-activity in the serum from normal rats given [^{75}Se]-methionine 20 hrs. previously can be located in the globulin component. In addition, when ^{75}Se-labelled proteins from an homogenate of washed trypanosomes and host plasma proteins were run in parallel on cellulose acetate, the results showed (Table 1.4) that the majority of trypanosome ^{75}Se-activity was associated with a band corresponding to the serum albumin of host plasma proteins/

Figure 1.1 Chromatographic separation on DEAE-cellulose of an homogenate of ^{75}Se-methionine labelled trypanosomes (⟵⟶) ^{75}Se radioactivity; (·····) absorbance 260 nm; (⟵⟶) absorbance 280 nm

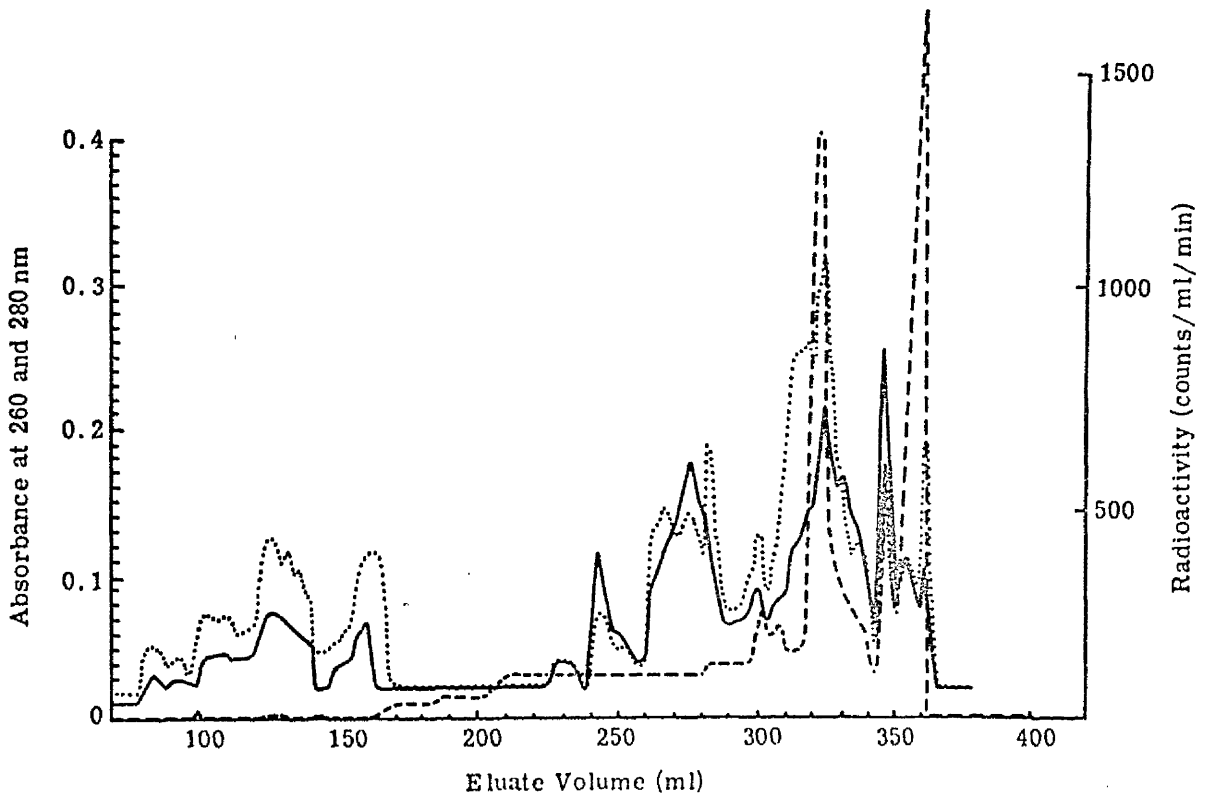


TABLE 1.3 Paper electrophoresis of serum proteins from a normal rat given 30 μCi of ^{75}Se -methionine 20 hrs. previously.

	cpm	cpm/mg protein	% total cpm	% total protein
Albumin	4	8	6	80
Globulin	58	446	94	20

TABLE 1.4 Cellulose acetate electrophoresis of ^{75}Se-labelled serum and trypanosome proteins.

Serum Protein faction	Serum % total cpm	Trypanosome % total cpm
Albumin	18.3	65.9
α-globulin	64.8	14.8
β-globulin	13.8	14.8
γ-globulin	3.1	4.4

proteins. In contrast, the majority of plasma protein ^{75}Se activity is again found in the globulin component, and in particular with the α and β globulins.

Spontaneous release of ^{75}Se-activity from radiolabelled trypanosomes: The following experiments were undertaken to ensure that ^{75}Se-activity was firmly bound to trypanosome protein and not readily eluted:-

(a) In vitro release of ^{75}Se-activity: In this experiment 5 ml. aliquots of culture medium in 20 ml. universal bottles were seeded with a known number of radiolabelled parasites, approximately 1 x 10^8 organisms. These bottles were then incubated at 37°C. for between 1 hr. and 4 hrs. Each sample was set up in quadruplicate. After incubation the trypanosomes in each sample were pelleted in a temperature controlled centrifuge (4°C. at 1000 g for 20 min). After this 1 ml. of supernate was removed and transferred to a separate counting vial. The radioactivity of both supernate and pellet was then determined. The release of activity is expressed as a percentage of the total c.p.m. of the sample, calculated thus:

$$\frac{5 \times \text{supernate c.p.m. in 1 ml.} \times 100}{\text{total c.p.m. of pellet and supernate}}$$

The results presented in Table 1.5 show that in vitro leakage of ^{75}Se-activity is approximately 10% after 4 hrs.

(b) In vivo release of ^{75}Se-activity: The release of ^{75}Se-activity into the supernate during in vitro incubation was found to be acceptable. To investigate the in vivo release of activity the following experiment was undertaken:- A group of normal mice received approximately 1 x 10^8 radiolabelled trypanosomes. These animals were then killed 1 hr. or 4 hrs. later and the radioactivity of a blood sample determined. The specific activity of radiolabelled trypanosomes was determined from washed separated trypanosomes obtained by DEAE cellulose chromatography.

The/

TABLE 1.5 Elution of radioactivity during in vitro incubation of ^{75}Se-labelled T. brucei.

Time (hrs)	% release of ^{75}Se-activity				
	Tube 1	Tube 2	Tube 3	Tube 4	$\bar{x}$
1	3.7	3.7	2.8	5.1	3.8 ± 0.5
2	5.6	4.8	6.3	5.8	5.6 ± 0.3
3	10.7	12.6	8.9	9.7	10.4 ± 0.8
4	9.8	8.5	13.0	8.9	10.1 ± 1.0

TABLE 1.6 The estimated total blood radioactivity 1 hr. and 4 hrs. after the injection of ^{75}Se-labelled T. brucei.

Time	Estimated total blood activity c.p.m.	% injected activity
1 hr.	3148 ± 109	100
4 hrs.	3096 ± 80	98

TABLE 1.7 The reduction in specific activity of 10^8 trypanosomes during in vitro incubation.

Time	c.p.m./10^8 trypanosomes	% injected activity
1 hr.	3860 ± 156	100
4 hrs.	2600 ± 193	67.4

The results presented in Table 1.6 show that the total blood c.p.m. based on an estimated blood volume did not fall significantly over the 4 hr. period. Thus the majority of the trypanosomes would appear to remain in the circulation over this period. There is however a fall in the specific activity of labelled trypanosomes over this period. Table 1.7 shows that the specific activity falls to 67.4% of the original activity of the trypanosomes injected. This fall is not associated with trypanosome removal from the circulation as the previous results show that estimates of total blood c.p.m. remain at approximately 100% over this period of 4 hours. The decrease in specific activity may be the result of parasite replication; indeed such a reduction would represent a parasite doubling time of approximately 6.5 hrs.

Re-utilisation of ^{75}Se-labelled metabolites by trypanosomes: The previous results show that there was a decrease in the specific activity of labelled trypanosomes in vivo. To ensure that any ^{75}Se-activity that may be released into the plasma was not re-utilised by circulating trypanosomes the following experiment was undertaken:-

An homogenate prepared from ^{75}Se-labelled trypanosomes (4328 ± 67cpm) equivalent to 1 x 10^8 trypanosomes was injected i.v. into rats with fulminating infections.

The results demonstrated that trypanosomes recovered after 1 hr. from rats injected with the homogenate of ^{75}Se-labelled trypanosomes did not incorporate a significant amount of the available activity (5 ± 2 c.p.m./10^8 trypanosomes).

From the results presented above it was decided to standardise the labelling procedure as an injection of 50 μCi of [^{75}Se]-methionine followed 20 hrs. later by DEAE cellulose chromatograph of the infected blood.

Tissue/

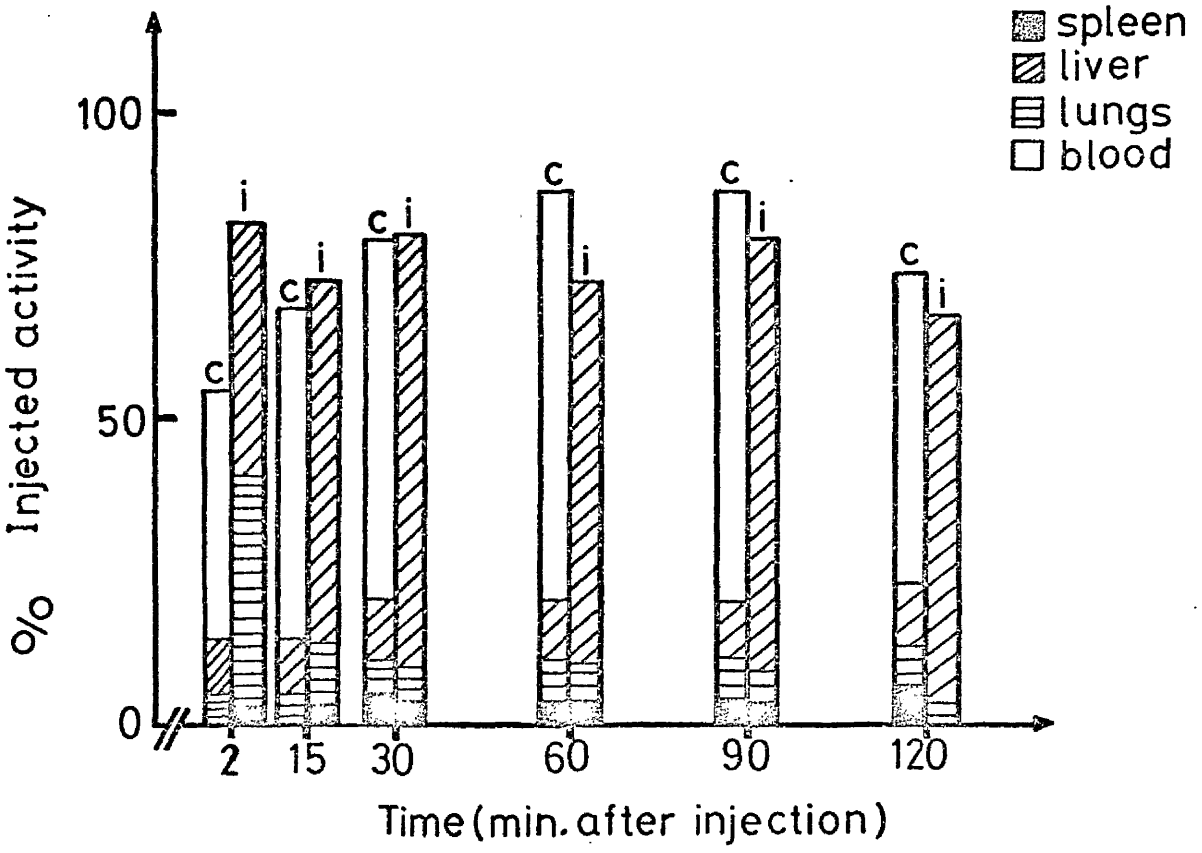
Tissue distribution of ^{75}Se-labelled trypanosomes at various times in normal and immunised mice:

Having established that no appreciable loss of ^{75}Se-activity occurred over 4 hrs. either *in vitro* or *in vivo*, the distribution of labelled trypanosomes in normal mice and mice immunised against the particular variant used in these experiments was measured at 2, 15, 30, 60, 90 and 120 mins. In particular, the radioactivity of the spleen, liver, lungs, kidneys, heart and blood was measured.

The tissue distribution of ^{75}Se-labelled *Trypanosoma brucei* at various times after their injection into mice is shown in Fig. 1.2. The most striking feature was the rapid reduction in ^{75}Se-activity from the blood of immune mice, i.e. < 3% compared to 67% of the injected activity in normal mice. This reduction in blood activity was associated with a rise in hepatic ^{75}Se-activity. The transient ^{75}Se-activity at 2 and 15 mins. after the injection of radiolabelled parasites in the lung of normal mice is thought to reflect the passage of trypanosomes through the pulmonary circulation. This is in contrast to the continual rise in the hepatic ^{75}Se-activity of immune mice which reaches a maximum value at 30 mins. after the injection of radiolabelled trypanosomes, i.e. approximately 65% after which time the values remain at this level.

The tissue distribution of ^{75}Se-labelled trypanosomes in normal mice does not alter over the time course of the experiment. Furthermore, the hepatic uptake is considerably less than is seen in the immunised mice, i.e. 9% compared to 65% and this is parallelled by a high blood activity. It would therefore appear that the hepatic localisation of ^{75}Se-activity is closely associated with the immune response of the host and probably reflects labelled trypanosomes being retained within the hepatic vasculature. The increase in hepatic uptake seen in immune animals is not a reflection of hepatomegaly as in these animals the liver/

Figure 1.2 The tissue distribution of ^{75}Se-labelled trypanosomes at intervals after injection into immunised (i) and control (c) mice.



liver weight, when expressed as a percentage of the total body weight, was 7.1 ± 0.2 compared to 6.3 ± 0.2 in control animals. Similarly, there was no significant degree of splenomegaly in immune animals, i.e. 0.9 ± 0.1 compared to 0.6 ± 0.1.

In general the tissue distribution of ^{75}Se-labelled T. brucei in the kidney and heart of both immune and normal mice was less than 3%.

The tissue distribution of ^{75}Se-activity following the injection of free [^{75}Se]-methionine into normal and immune mice:

To confirm that the radioactivity values obtained in the previous experiment were not the result of increased hepatic uptake of free [^{75}Se]-methionine in immune mice the following experiment was undertaken.

A group of normal mice and a group of immune mice were given 10 μCi of free [^{75}Se]- methionine by i.v. injection and 1 hour later the tissue distribution of ^{75}Se-activity recorded. Table 1.8 shows that the tissue distribution of [^{75}Se]-methionine 1 hr. after its injection into normal and immune mice is the same. The majority of the activity is located in the liver. However this can be explained as a consequence of both the size of the liver when compared to the spleen and also to the importance of the liver in protein biosynthesis.

The hepatic activity although higher than in normal mice given ^{75}Se-T. brucei does not approach the values of hepatic uptake seen in immune mice.

TABLE 1.8 Tissue distribution of [^{75}Se]- methionine in normal and immune mice.

	tissue distribution (% injected activity)	
	spleen	liver
Control	< 3.0	16.5 ± 1.6
Immune	< 3.0	15.7 ± 1.1

Discussion.

The major use of [^{75}Se] seleno-methionine (Blau and Bender, 1962) has been in tumour detection of the pancreas and parathyroid It has, however, been more recently used in protein turnover studies (Waterlow, Garrow and Millward, 1969).

Methionine is important in animal protein biosynthesis because of their failure to use H_2S or SO_4^{2} in the formation of cysteine which is an essential amino-acid. In addition, methionine is the starting amino-acid for all proteins in the form of N-formyl methionine. Indeed the formation of tRNA in the nucleoplasm is essential to the formation of proteins. Therefore the formation of an amino-acid analogue, [^{75}Se-] methionine, which has been shown to be readily incorporated into newly synthesised proteins was clearly of great experimental value (Awwad et al,1966).

Its application to the radiolabelling of parasites has until recently been limited to fascioliasis (Mulligan, Cuperlovic, Borojevic and Lalic, 1972) and schistosomiasis (Christensen, 1977) where the parasites were labelled in vitro. The ^{75}Se-labelled schistosomula were used to follow the migration of this parasite through the host. [^{75}Se]-methionine has also been used in gut metabolite studies during the immune expulsion of Nippostrongylus brasiliensis (Maclean, 1977). The present study has shown that [^{75}Se]-methionine can be used effectively to radiolabel African trypanosomes. These radiolabelled parasites can be used successfully for in vivo clearance studies. A possible disadvantage of the technique is the low specific activity of the trypanosomes and this obviously limited their use to situations where relatively large numbers of parasites can be used. However, the present technique has several important advantages over previously described techniques in that it fulfils most of the criteria for a trace label - (a) the organisms are still/

still viable and pathogenic; (b) there is an insignificant and accountable release of ^{75}Se-activity over several hours; (c) as a gamma-emitting isotope, with a half life of 120 days [^{75}Se]-methionine is easily counted with the minimum of sample preparation; and (d) the relatively short labelling period enables specific populations of trypanosomes to be labelled.

The preparation of labelled trypanosomes is apparently dependent both on the time at which the isotope is given during an infection and on the quantity of isotope used. In order for the parasite to take up sufficient isotope to become usefully labelled, they must be present in high numbers. Failing this requirement, as has been shown by Awwad et al (1966), the [^{75}Se]-methionine is rapidly removed from the circulation and incorporated into plasma proteins. This is given some support by the failure of trypanosomes, incubated with the isotope over the duration of the infection, to take up significant levels of isotope. It is likely that the [^{75}Se]-methionine had been incorporated into plasma proteins before the parasites were present in sufficient numbers. It is also of interest to note that the failure of these parasites, incubated over the duration of infection, to take up isotope may imply indirectly that pinocytosis of plasma proteins, if it occurs, in bloodstream trypanosomes (Brown, Armstrong and Valentine, 1965; Langreth and Balber, 1975) does not contribute to the labelling. The ability of the quantity of isotope administered to influence the specific activity gives some support to the isotope's metabolic incorporation. Furthermore, approximately 95% of the trypanosome activity can be precipitated with 10% T.C.A., showing that the [^{75}Se]-methionine is firmly bound to newly synthesised protein.

It has already been suggested that pinocytosis of macromolecules does/

does not play an important role in the labelling but it was important to clarify the ability of the parasites to adsorb labelled host plasma protein on to their surface. This has been shown to occur by several authors (Ketteridge, 1970; Vickerman, 1972; Seed, 1974 and Diffley, 1978) and the surface labelling that may result from the adsorption of [^{75}Se]-methionine labelled host plasma proteins on to the surface of trypanosomes may be easily removed and limit the technique's applications. Although 95% of ^{75}Se-trypanosome activity could be precipitated using T.C.A., it did not differentiate between trypanosome-bound activity and host plasma protein activity adsorbed on to the parasite surface. The ^{75}Se-activity of trypanosomes was confirmed to be associated with trypanosomal protein by both DEAE cellulose chromatography and by electrophoresis. DEAE cellulose chromatography showed that the ^{75}Se-activity was associated with several protein peaks originating from trypanosomes rather than from host serum proteins. This association was confirmed by electrophoresis in which the ^{75}Se-trypanosome homogenate was run in parallel with serum proteins. This showed that the majority of the trypanosome activity migrated at the same rate as the serum albumin band, whilst the latter contained little ^{75}Se-activity. In contrast, the majority of serum protein activity was associated with its globulin bands. As the donor rats used to produce labelled parasites were always sub-lethally irradiated, it is unlikely that a significant amount of host globulin as antibody would be taken up as label. It is therefore improbable that the [^{75}Se]-methionine labelling of host plasma proteins, and in particular globulins, plays a significant role in the [^{75}Se-] methionine labelling of trypanosomes. Also Awwad *et al* (1966) have shown that incubation of serum proteins *in vitro* with [^{75}Se] -methionine does not produce labelled proteins, thus further supporting its incorporation during the biosynthesis of plasma proteins.

In/

In support of findings presented in this chapter, Black and Hewitt (1980) have recently labelled T. brucei in vitro with [^{75}Se]-methionine, in the absence of serum proteins, and have achieved high specific activities.

If the parasites from the present experiments are to be used for immunological clearance studies, it is important that the ^{75}Se-activity remain firmly bound to the trypanosome. The results show that trypanosome-bound ^{75}Se-activity does remain firmly bound both in vitro and more importantly in vivo. The release of activity in vivo is higher than in vitro, although this would not be unexpected. During the parasite's passage through the circulation of the host some parasites may be destroyed by mechanical damage. It is also expected that some activity may be lost as a result of surface coat secretion (Weitz, 1960; Macadam and Herbert, 1970b) although Black and Hewitt (1980) have shown that no appreciable loss of surface coat occurs in their system. The most probable cause for the reduction in specific activity is parasite replication. Such a loss of activity would correspond to a doubling time of approximately 6.5 hrs.

It was important to ensure that any apparent loss of ^{75}Se-activity from the parasites was not re-utilised by other circulating parasites. The injection of homogenised ^{75}Se-labelled trypanosomes into mice with fulminating infections in an attempt to label the circulating trypanosomes clearly demonstrated that little re-utilisation of ^{75}Se-metabolites occurs. This would not be entirely unexpected in a system with a labelling efficiency of 0.04%.

The tissue distribution of ^{75}Se-labelled trypanosomes in normal and immune mice demonstrated the value of this technique in immunological studies. Whereas normal mice retain the majority of injected ^{75}Se-labelled T. brucei in their circulation, they rapidly disappear from the circulation of immune mice. The principal site of this removal was the liver. The transient/

transient uptake in the lungs of immune mice, but not in normal mice, is thought to represent trypanosomes in the pulmonary circulation. It is, however, possible that it may reflect a sensitization of the macrophages in the lung to trypanosome antigen.

^{75}Se-labelled trypanosomes clearly have several advantages when compared with previously reported techniques in exploring the role of macrophages and antibody in the immune response to trypanosomes. For example, while foreign red cells, colloidal carbon and various other colloids can be used to measure phagocytic function in infected mice (Souhami, 1972; Murray et al, 1974a), these results do not necessarily reflect the host's ability to remove trypanosomes from the circulation. Tests of antibody function such as agglutination, complement fixation and trypano- lysis, are subject to the similar criticisms. In contrast, the removal of labelled parasites from the circulation provides a direct index of the host's capacity to eliminate specific populations of trypanosomes. Furthermore, it enables the site of phagocytosis to be determined with certainty.

Recently Ferrante and Jenkin (1978) using [^{32}P] orthophosphate in an *in vitro* system and Stevens and Moulton (1978) using [^{3}H]-adenine in an *in vivo* labelling technique, have incorporated these isotopes into *T. lewisi* and *T. brucei* respectively. The former have measured the tissue distribution of ^{32}P-labelled *T. lewisi* and confirmed the importance of the liver in parasite removal, while both show the importance of a specific immune response in macrophage uptake of trypanosomes.

In conclusion, the incorporation of [^{75}Se]-methionine has been shown to be a reliable and simple technique for the *in vivo* labelling of *Trypanosoma brucei*. In addition, ^{75}Se-labelled *T. brucei* have been successfully used to study the fate of circulating parasites in immune and normal mice, and have demonstrated the importance of the liver in trypanosome removal.

Chapter 2

MECHANISMS IN IMMUNE ANIMALS

Introduction.

The immune response that follows natural infection with African trypanosomes is only occasionally totally effective against the parasite (Fiennes, 1970). This is reflected in the variety in both the pattern and the course of natural and experimental infections. The principal reason for the failure of the host's immune response is thought to result from the parasites' ability to undergo antigenic variation (Ritz, 1916; Lourie and O'Connor, 1937; Gray, 1965a; Vickerman, 1969). Thus while the host is maintaining an effective immune response against the predominant variant, a new variant or variants emerge to repopulate the blood and the cycle continues.

An effective immunity against a particular antigenic variant can be readily developed by a variety of vaccination procedures (General Introduction; Terry, 1976; Murray & Urquhart, 1977). Such immunity is however only directed against the particular variant used for the vaccine, and does not result in protection against heterotypes. This immunity is generally thought to depend on serum antibody. Thus humoral responses to specific antigenic variants have been demonstrated *in vitro* by a variety of methods (General Introduction). Furthermore, passive transfer of protection has been reported using immune serum (Seed & Gam, 1966). Indeed recently Campbell and Phillips (1976) have shown that resistance to infection can be transferred by B-lymphocytes but not by T-lymphocytes. It has also been reported that mice injected from birth with anti-μ chain serum (Campbell, Esser & Weinbaum, 1977) and thus deficient in B-lymphocytes were incapable of controlling the initial parasitaemia. These mice also failed to respond to an irradiated trypanosome vaccine. The importance of immuno-competent B-lymphocytes would therefore appear to be a prerequisite for the production of protective immunity.

There/

There is also evidence that macrophages may play an important role in protection against trypanosomes, particularly in the presence of homologous antiserum (Lumsden & Herbert, 1967; Goodwin, 1970; Takayanagi, Nakatake & Enriques, 1974). The presence of Fc and C3 receptors on the surface of macrophages (Mantovani, Rabinovitch & Nussenzweig, 1972; Hopf, Buschenfelde & Dierich, 1976) may enable the attachment of the parasite to the macrophage prior to phagocytosis. Takayanagi and Nakatake (1977) have shown that the Fc portion of antitrypanosomal antibody is responsible for parasite attachment to peritoneal macrophages in vitro.

Activation of macrophages during trypanosome infections has also been suggested (Murray et al, 1974b; Corsini et al, 1977). Indeed it has been reported that activation of macrophages occurs during several protozoan infections (Salaman, Wedderburn & Bruce-Chwatt, 1969; Strickland, Pettitt and Voller, 1973). Similarly, activated macrophages can be produced by various agents such as Mycobacterium bovis (Blanden, Lefford and Mackaness, 1969) or Corynebacterium parvum (Otu, Russel and White, 1977). Such treatment produces hypertrophy and hyperplasia of the mononuclear phagocytic system (MPS) and results in an increased phagocytic index. Similar alterations have been noted during T. brucei infections (Murray et al, 1974c) and hepato-splenomegaly may partly be a result of macrophage activation.

Finally, the role of complement in immune clearance is still unresolved. Hypocomplementaemia, attributed to complement activation by the parasites, occurs in trypanosome infections and has been suggested as a mechanism whereby the parasites may avoid complement-dependent trypanolysis (Nielsen & Sheppard, 1977; Musoke & Barbet, 1977), although complement also has been implicated in the generation of an immune response and formation of immunological memory (Klaus & Humphrey, 1977) by promoting the location of antigen in germinal centres and in opsonisation of antigen prior to their phagocytosis (Pepys, 1976).

This/

This chapter details the results from a series of experiments designed to investigate the mechanisms involved in the removal of circulating parasites. In particular, the roles and relative importance of antibody, macrophage activation and complement were investigated.

Materials and Methods.

Parasite: The stabilate of T. brucei used was derived from a stock of TREU 226.

Experimental Animals: Female Hooded Lister rats, female CFLP mice and AKR strain mice genetically deficient in the fifth component of complement (C5) were used. Prior to their use the absence of C5 in AKR mice was confirmed by haemolytic radial diffusion (Lachman and Hobart, 1978).

Radiolabelling Technique: Radiolabelled trypanosomes were prepared by i.v. injection of 50μCi ^{75}Se -methionine into rats with fulminating parasitaemias. The labelled trypanosomes were separated from blood cells 20 hrs. later by DEAE-cellulose chromatography (General Materials and Methods).

Injection of Labelled Trypanosomes and Sampling: The suspension of washed labelled trypanosomes was diluted with PBGS to give an injection dose of 1 x 10^8 parasites per mouse (approximately 4000 c.p.m.).

One hour after i.v. injection of labelled parasites the mice were given an overdose of trichloroethylene anaesthesia and the distribution of radiolabelled parasites measured in spleen, liver and blood (General Materials and Methods).

Immunisation of Mice:

(a) Mice were immunised by infection and drug treatment on day 4 of infection (General Materials and Methods); and (b) mice were passively immunised by an i.v. injection of 0.2 ml. of undiluted HIS 15 mins. after the injection of ^{75}Se-labelled trypanosomes.

Irradiation/

Irradiation of Experimental Animals: When necessary mice and rats were sub-lethally irradiated 1 day prior to infection with 650 rad in a ^{60}Co source.

Decomplementation of Mice: Mice were decomplemented by three i.v. injections of purified cobra venom factor (CVF) over a period of 24 hrs. Each mouse received a total of 25 µl in three 8-hourly injections. The ability of these mice to remove radiolabelled trypanosomes was determined 24 hrs. after the final injection of CVF. Serum C3 levels were quantified by rocket immunoelectrophoresis (Laurel, 1966) using rabbit anti-mouse C3 (Nordic Immunological Reagents Ltd., Berks.).

Stimulation of Mononuclear Phagocytic System: Non-specific stimulation of the mononuclear phagocytic system (MPS) was achieved by i.v. injection of either Mycobacterium bovis (BCG vaccine, Glaxo), 1.5×10^7 organisms per mouse or Corynebacterium parvum (Coparvax, Wellcome) 1.4 mg. per mouse, 10 days prior to clearance studies.

Preparation of Hyperimmune Serum: Hyperimmune serum (HIS) was prepared in rats by infection and treatment, followed by two subsequent trypanosome challenge injections (General Materials and Methods).

Implantation of Millipore Diffusion Chambers: Millipore diffusion chambers were assembled from plexiglas rings (14 mm. dia.) and Millipore filters (0.4 µm pore size) both supplied by Millipore U.K. Ltd. (London).

Separated, washed trypanosomes were injected into the diffusion chamber through a hole in the plexiglas ring. This hole was then sealed with a piece of plastic thread and cemented in place.

Two diffusion chambers were then inserted into the peritoneal cavity of each immune rat or normal rat via a small incision on the ventral abdominal midline. The peritoneal wall was closed by two silk sutures and the abdominal skin closed using two suture clamps.

The laparotomy was performed under neuroleptanalgesic sedation using a mixture of Fentanyl base and Fluorisone (Hypnorm, Janssen Pharmaceutica/

Pharmaceutica, Belgium) together with diazepam (Valium, Roche Products Ltd., Welwyn Garden City).

The animals were maintained in an anaesthetised state for 20 hrs. after which time they were exsanguinated and the chambers removed.

In vitro Treatment of Trypanosomes with Serum:

(a) Neutralising antibody infectivity tests were performed on sera (Lumsden et al, 1973), as detailed in General Materials and Methods.

(b) Agglutination titres of fresh HIS were determined in microtitre plates, as described in General Materials and Methods.

(c) Clearance studies using labelled trypanosomes were conducted following their in vitro treatment with serum. This consisted of a modified infectivity neutralisation test in which ^{75}Se-labelled trypanosomes were incubated at 4°C. for 30 min. in HIS or NMS. Some of the trypanosomes were then washed three times with PBGS.

Groups of mice were inoculated i.v. with a suspension containing 1×10^8 trypanosomes as described above.

Homogenisation of Trypanosomes: Trypanosomes were homogenised by alternate freezing and thawing as previously described (General Materials and Methods).

Silica Dust Poisoning of the MPS: Silica dust (DQ 12 Quartz, <5 μm particle size) was suspended in PBS. Groups of normal mice received either 2, 4, 6, 8 or 12 mg. of silica 4 hrs. before the injection of ^{75}Se-labelled trypanosomes. The silica dust was kindly donated by Dr. I.M. Reisner, Steinkohlenberg Bauerein, West Germany.

In vivo adsorption of antitrypanosome antibody: Unlabelled T. brucei 226, prepared by DEAE cellulose chromatography were injected i.v. into mice 15 mins. prior to the injection of ^{75}Se-labelled trypanosomes.

Results./

Results.

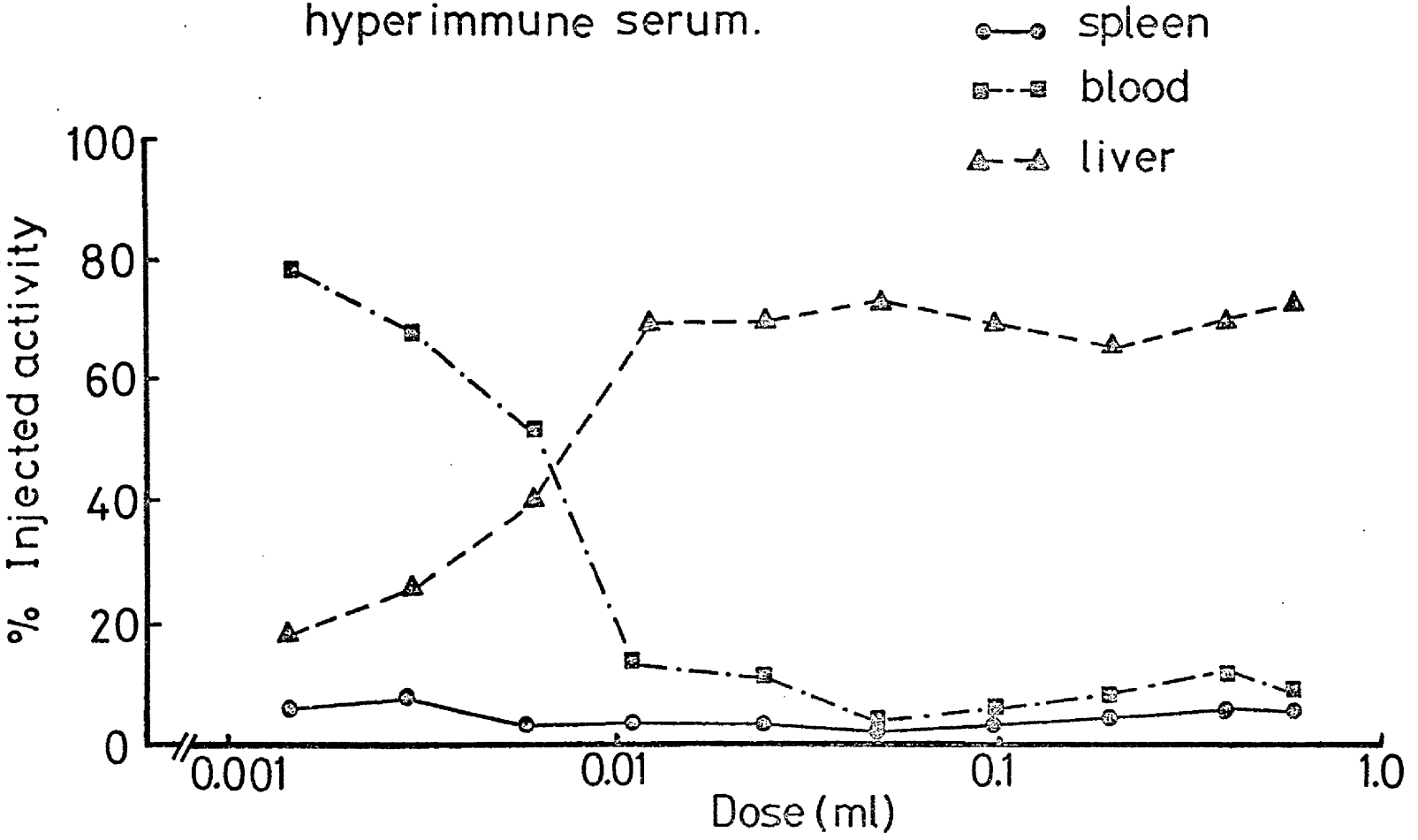
The Effect of Hyperimmune Serum on Clearance of ^{75}Se-labelled T. brucei in Normal Mice:

The rapid removal of ^{75}Se-labelled T. brucei from the circulation of immune mice, demonstrated in the previous chapter, was principally achieved by the liver. The role of antibody in this process was investigated both in vivo and in vitro using HIS prepared in rats. Firstly the effect of HIS was assessed in vivo by injecting normal mice with labelled trypanosomes followed 15 mins. later by various i.v. doses of HIS. The results presented in Fig. 2.1 show that a level of circulating antibody could readily be obtained by passive immunisation which facilitated hepatic uptake of trypanosomes to a degree similar to that of actively immunised mice. Indeed only very small quantities of HIS were required to produce this uptake, i.e. an injection of HIS greater than 0.01 ml. produces approximately 60% hepatic uptake.

By injecting whole serum i.v. it is difficult to assess whether hepatic uptake occurs as a result of opsonisation of the trypanosome or is mediated through cytophilic antibody attached to macrophages in the liver. Thus the effect of pre-treatment of labelled trypanosomes with HIS in vitro prior to their injection into normal mice was investigated. In an attempt to differentiate between opsonic and cytophilic antibody, unbound HIS was removed by washing the trypanosomes in PBGS three times prior to their i.v. injection into normal mice. The results presented in Table 2.1 clearly indicate that such treatment can induce levels of hepatic uptake which were similar to those of either actively or passively immunised mice. Furthermore, washing free HIS from the trypanosomes prior to their injection did not diminish the hepatic uptake.

Macrophage/

Figure 2.1 The tissue distribution of ^{75}Se-labelled trypanosomes in mice passively immunised with various doses of hyperimmune serum.



Macrophage Function in the Absence of Antibody: The previous experiment showed that hepatic macrophages without experience of trypanosome antigen prior to the injection of labelled trypanosomes were as efficacious in trypanosome removal in the presence of passively acquired HIS as those of actively immunised mice. It was thought useful however to investigate the phagocytic efficiency of antigen-experienced macrophages in the absence of antibody. In this experiment groups of mice were immunosuppressed by sub-lethal irradiation with 650 rad. One day later these mice and normal controls were infected. After three days the animals were cured with Berenil and a further 4 days later injected with ^{75}Se-labelled T. brucei.

The results presented in Table 2.2 show that the immunisation procedure of infection and chemotherapy resulted in high hepatic clearance of trypanosomes which was not a drug induced artefact, as mice given only Berenil failed to produce these high hepatic uptakes. However, those mice which were irradiated prior to their infection had markedly reduced clearance values to levels only slightly above those of normal mice.

There are two possible explanations for this result; first, prior irradiation may have damaged the phagocytic ability of the macrophages. Secondly, irradiation may have prevented the production of effective antibody levels.

The first possibility was examined in a group of 20 normal mice, some of which were irradiated with 650 rad and 8 days later injected with ^{75}Se-labelled trypanosomes followed 15 mins. later of 0.2 ml. of a 1:8 dilution of HIS. The results presented in Table 2.3 show that prior irradiation does not impair the phagocytic function of hepatic macrophages and therefore does not affect hepatic uptake.

The absence of antitrypanosomal antibody was confirmed by trypanosome agglutination tests. In such tests pooled sera from irradiated mice failed to produce agglutination of trypanosomes either before/

TABLE 2.1. The effect of in vitro treatment with hyperimmune serum on the tissue distribution of ^{75}Se-labelled trypanosomes in normal mice.

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
HIS	<3.0	59.4 ± 1.8	<3.0
HIS washed	<3.0	64.6 ± 3.7	<3.0
NMS	<3.0	9.5 + 0.5	69.2 + 5.6
NMS washed	5.2	11.0 + 0.3	69.2 ± 4.2

TABLE 2.2. The effect of prior irradiation on the tissue distribution of ^{75}Se-labelled trypanosomes in actively immunised and normal mice.

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
irradiated immune	3.8 ± 0.3	13.2 ± 3.1	55.3 ± 7.7
non-irradiated immune	<3.0	49.3 ± 1.8	<3.0
irradiated drug control	4.7 ± 1.7	8.3 ± 0.6	67.6 ± 8.9
non-irradiated drug control	4.5 ± 0.3	9.2 ± 0.3	73.9 ± 5.7
irradiated control	<3.0	8.3 ± 0.5	61.7 ± 6.0
non-irradiated control	3.2 ± 0.6	6.5 ± 0.7	68.5 ± 2.9

before or after Berenil therapy. In contrast, similar non-irradiated mice treated with Berenil produced a strong agglutination of the parasite, approximately $^{1}/_{128}$.

The Effect of MPS Activation on Phagocytic Removal of ^{75}Se-labelled Trypanosomes:

Since expansion and the activation of the MPS is a characteristic feature of trypanosome infected animals (Murray et al, 1974c; Stevens and Moulton, 1978), it is possible that apart from antibody induced clearance, trypanosomes may also be removed from the circulation in such animals by a non-specific phagocytosis.

In order to test this hypothesis the clearance of trypanosomes from the circulation was measured in normal mice which had previously received either BCG or C. parvum as non-specific modulators of the MPS. Both have been shown to cause increases in liver and spleen weights associated with hyperactivity of their macrophages and increased phagocytosis of carbon particles and bacterials cells (Stiffel, Mouton and Biozzi, 1970).

The results presented in Table 2.4 show that prior treatment with either BCG or C. parvum was associated with increases in hepatic and splenic weights. This was reflected by proportionate increases in trypanosome uptake by both organs. However, the level of hepatic uptake was very much less than that obtained with HIS, i.e. less than 11% compared to approximately 60% (see Fig. 2.1).

Role of Complement in Immune Clearance: The experiments so far described have clearly demonstrated that antibodies are essential in inducing blood clearance of trypanosomes. However, these results do not show whether whole opsonised trypanosomes can be removed or if a complement dependent mechanism is an essential pre-requisite for phagocytosis. In order to investigate this latter possibility a series of experiments was conducted.

Millipore/

TABLE 2.3. The effect of prior irradiation on the tissue distribution of ^{75}Se-labelled trypanosomes in passively immunised and normal mice.

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
irradiated immune	<3.0	77.8 ± 1.6	<3.0
non-irradiated immune	<3.0	67.4 ± 2.4	<3.0
irradiated non-immune	<3.0	14.3 ± 1.2	80.6 ± 5.9
non-irradiated non-immune	<3.0	11.0 ± 0.7	77.5 ± 3.2

TABLE 2.4. The effect of non-specific stimulation of the MPS on the tissue distribution of ^{75}Se-labelled trypanosomes.

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
BCG	5.5 ± 0.5 (0.81 ± 0.15)*	8.8 ± 0.5 (6.99 ± 0.12)	63.9 ± 2.9
C. parvum	13.3 ± 1.1 (2.25 ± 0.11)	10.2 ± 0.7 (13.15 ± 0.37)	49.3 ± 2.6
non-treated	3.2 ± 0.65 (0.37 ± 0.02)	6.5 ± 0.7 (6.27± 0.21)	68.5 ± 2.9

* figures in parenthesis are organ weights in grams.

Millipore diffusion chambers.

The effect of immune serum on trypanosomes was investigated in vivo in an experiment in which trypanosomes enclosed within Millipore diffusion chambers were maintained in the peritoneal cavity of immune rats and normal rats. The results indicated that when 1 x 10^8 ^{75}Se-labelled T. brucei were maintained for 20 hrs. in diffusion chambers they appear as an agglutinated mass of cells in the immune rats. In contrast, trypanosomes maintained within the peritoneal cavity of normal rats remain freely motile. When radioactivity measurements were made before and after implantation, although the trypanosomes were agglutinated by the immune rat they retained their radioactivity. Thus lysis was not observed either directly or by release of ^{75}Se-activity from the diffusion chamber. In addition, the agglutinated trypanosomes from immune rats were shown to be non-infective by infectivity neutralisation tests (100% survival in 5 mice receiving an aliquot of the pooled contents of the diffusion chambers). In contrast, trypanosomes from diffusion chambers maintained in normal rats retained their infectivity (100% mortality in 5 recipient mice).

Immune Clearance in C5-deficient and C3-depleted mice: The in vivo clearance ability of passively immunised mice, which were either genetically deficient in C5 or depleted of C3 by prior treatment with CVF was measured. The former mice permit an investigation of the terminal lytic pathway of complement activation, whereas the latter should reveal the role of C3 opsonisation in immune clearance. Immune clearance was measured in passively immunised mice by i.v. injection of ^{75}Se-labelled T. brucei followed 15 mins. later by an injection of 0.2 ml. HIS. Groups of mice received either undiluted serum or a 1:8 dilution in PBGS. The results presented in Table 2.5 show that C5 deficient mice had hepatic uptakes only slightly lower than those of passively immunised normal/

TABLE 2.5. The tissue distribution of ^{75}Se-labelled trypanosomes in passively immunised normal C5 deficient and C3 depleted mice.

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
C5 deficient 1:8 HIS	<3.0	54.7 ± 3.1	20.8 ± 3.6
C5 deficient	<3.0	11.1 ± 1.3	73.7 ± 5.6
C3 depleted HIS	<3.0	74.0 ± 2.6	<3.0
C3 depleted 1:8 HIS	8.7 ± 0.8	10.1 ± 0.8	58.3 ± 3.2
Control HIS	<3.0	60.0 ± 2.1	<3.0
Control 1:8 HIS	<3.0	67.4 ± 2.5	<3.0
Control	6.4 ± 1.1	11.0 ± 0.7	77.5 ± 3.2

TABLE 2.6. The tissue distribution of ^{75}Se-labelled trypanosomes in actively immunised C3 depleted mice.

	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
C3 depleted immune	<3.0	79.2 ± 3.7	<3.0
Immune	<3.0	63.8 ± 3.4	<3.0
Control	7.9 ± 0.2	8.6 ± 0.2	68.1 ± 2.4

normal mice at both levels of HIS administration. However, in passively immunised C3-depleted mice in which C3 was reduced by 75% of normal circulating levels, two levels of hepatic uptake were observed. Those mice receiving undiluted HIS gave hepatic uptakes similar to passively immunised normal mice. However, in mice receiving 0.2 ml. of a 1:8 dilution of HIS, the hepatic uptake remained at non-immunised levels.

Immune Clearance in Actively Immunised C3-depleted Mice: Immune clearance was measured in actively immunised mice depleted of C3 by CVF treatment. The results presented in Table 2.6 show that actively immunised mice can achieve high hepatic uptake even in the absence of C3, i.e. approximately 64% and 79% respectively compared to control value of approximately 9%.

The Effect of MPS Poisoning on Immune Clearance of ^{75}Se-labelled Trypanosomes

It has already been shown that macrophages in the absence of antibody are unable to effect the removal of ^{75}Se-labelled trypanosomes from the circulation. To investigate whether the removal of ^{75}Se-*T. brucei* was also dependent upon an intact and functioning MPS, the tissue distribution of radiolabelled parasites was assessed in groups of passively immunised mice which had received various doses of silica dust. Silica is known to decrease dehydrogenase activity and thus interfere with phagocytosis (Marks and Nagelschmidt, 1959; Allison, 1970).

The results presented in Table 2.7 show that passively immunised mice receiving silica dust have a reduced hepatic uptake when compared to untreated immunised mice, i.e. approximately 35% compared to approximately 60%. Furthermore the reduction in hepatic uptake is to a certain degree independent of the quantity of silica given and the reduction in hepatic uptake never falls to normal levels but remains elevated, i.e. 35% compared to 9%.

The/

TABLE 2.7. The effect of a single i.v. injection of silica dust on the tissue distribution of ^{75}Se-labelled trypanosomes in passively immunised mice.

Quantity of silica injected into immune mice (mg)	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
0	<3.0	55.7 ± 1.4	5.3 ± 0.8
2	<3.0	38.6 ± 5.9	9.0 ± 1.1
4	5.0 ± 0.8	32.2 ± 3.3	14.4 ± 2.8
6	3.3 ± 0.8	36.2 ± 4.5	16.8 ± 5.3
8	3.3 ± 0.6	34.3 ± 0.5	14.1 ± 3.4
12	<3.0	20.7 ± 2.4	25.5 ± 11.1
non-immunised	6.6 ± 0.7	10.5 ± 0.4	71.7 ± 4.0

TABLE 2.8 The tissue distribution of ^{75}Se-labelled trypanosomes in actively immunised mice after the injection of unlabelled trypanosomes and following passive immunisation with HIS.

	Tissue distribution (% injected activity)				
	Unlabelled parasites	HIS	Spleen	Liver	Blood
Immune	+	-	4.8 ± 0.6	15.5 ± 2.3	11.7 ± 2.3
Immune	-	-	2.8 ± 0.2	66.1 ± 1.8	<3.0
Immune	+	+	5.3 ± 1.6	63.2 ± 4.6	12.7 ± 1.7
Control	-	+	0.6 ± 0.1	53.6 ± 2.5	6.7 ± 1.6
Control	-	-	6.8 ± 1.4	16.6 ± 0.6	14.9 ± 0.5

The effect of unlabelled trypanosomes on the immune clearance of ^{75}Se-labelled trypanosomes:

The present results have shown the importance of antibody in the hepatic uptake of ^{75}Se-labelled T. brucei. To confirm this relationship, groups of immune mice were given an i.v. injection of 5×10^8 unlabelled trypanosomes 15 mins. prior to the injection of ^{75}Se-labelled T. brucei. Some of these groups also received an i.v. injection of 0.2 ml. of undiluted HIS 15 mins. after the injection of labelled trypanosomes.

The results presented in Table 2.8 show that unlabelled trypanosomes are able to prevent the hepatic uptake of labelled parasites. This is not due to macrophage blockade in antibody depleted mice as an injection of HIS restores the hepatic uptake of labelled parasites.

In addition the agglutination titre of mice injected with unlabelled parasites was reduced when compared to immune mice, i.e. approximately $^1/_{256}$ compared to zero. This result was confirmed by infectivity neutralisation tests. In such tests only serum from immune mice which did not receive an injection of unlabelled parasites conferred protection (i.e. 100% protection in a group of 5 mice).

It is therefore evident that trypanosomes can reduce the effective level of circulating antibody presumably by the adsorption of antibody on to their surface.

Discussion.

The results presented in this and the previous chapter show that the ability of immune mice to remove circulating trypanosomes is principally achieved by hepatic uptake. Thus by 60 mins. after the injection of labelled trypanosomes, the majority of the injected activity appears in the liver of immune animals, approximately 60% compared with approximately 8% in normal mice. In contrast, the majority of the activity in normal mice remains in their circulation.

In theory the removal of trypanosomes from the circulation may be achieved by the uptake of antibody-coated whole trypanosomes; particulate trypanosome antigen resulting from trypanosome disruption by immune lysis; cytophilic antibody present on the surface of the sinusoidal cells (Kupffer cells) as a result of previous exposure to trypanosome antigen; or, finally, as a result of an expanded and/or an activated MPS.

From the results shown in Fig. 2.1, it is apparent that the amount of passively acquired circulating antibody is closely related to the degree of hepatic uptake and that blood clearance levels as high as those observed in actively immunised mice can readily be achieved.

The fact that the activity retained by these organs does not completely account for all the injected activity suggests that some labelled trypanosomes are absent from the main systemic circulation. This may be either as a result of trapping in small capillaries (Goodwin, 1970) or of non-specific phagocytosis of low numbers of trypanosomes throughout the body.

The importance of antibody in the hepatic uptake of radiolabelled trypanosomes was confirmed following their exposure to HIS *in vitro*. On injection into normal mice, these pre-treated trypanosomes produced a degree of hepatic uptake similar to that of actively or passively immunised mice. Furthermore, washing unbound HIS from the trypanosome suspension prior to their injection failed to reduce their hepatic uptake. Thus antibody is apparently directed against the parasite and is presumably acting as an opsonin rather than as a cytophilic antibody on the surface of hepatic macrophages.

The minor contribution of macrophages activated by trypanosome infection or by immunostimulants in the hepatic uptake of radiolabelled trypanosomes/

trypanosomes was demonstrated in two studies in which macrophage activity in the absence of specific anti-trypanosome antibody was evaluated. In the first, the suppression of antibody production by prior irradiation at a level which did not impair phagocytic function abolished the ability of infected and treated mice to remove high levels of radiolabelled parasites. In the second, MPS activation by non-specific stimulants BCG and C. parvum only marginally increased clearance values above those of control animals, and this was closely correlated with the degree of hepatomegaly and splenomegaly induced by these agents.

However the macrophage is important in the removal of suitably opsonised parasites from the circulation and this process is probably dependent upon Fc and C3 receptors on the surface of the macrophage (Mantovani et al, 1972; Bianco, Griffin and Silverstein, 1975; Hopf et al, 1976; Alexander, Andrews, Leslie and Wood, 1978; Shaw and Griffin, 1981) which may bind exposed Fc and C3 components on the surface of circulating trypanosomes (Diffley, 1978; Diffley and Honigberg, 1978; Rickman and Cox, 1979).

Complement mediated lysis of trypanosomes did not appear to be important in the removal of circulating parasites. This was demonstrated both by the agglutination rather than lysis of parasites maintained within millipore diffusion chambers in the peritoneum of immune rats and by the ability of passively immunised C5-deficient mice to remove a radiolabelled trypanosome challenge. Thus the terminal lytic pathway does not appear to be required for trypanosome removal.

In contrast, at low levels of passive immunisation of C3-depleted mice, i.e. 1:8 dilution of HIS, C3 appears to be required to initiate hepatic uptake. No such correlation existed at high levels of passive immunisation, i.e. undiluted HIS, or in actively immunised C3 depleted mice.. C3 and its degradation products are known to be opsonic and the localisation/

localisation of these components on to the surface of antigens is known to promote their attachment to C3 receptors on the surface of macrophages (Pepys, 1976; Shaw and Griffin, 1981). Thus, it is therefore possible that at low antibody titre, C3 may have an important role in increasing the opsonic capability of the available antibody, facilitating trypanosome removal via C3 as well as Fc receptors.

There are clearly two stages to hepatic uptake. First, a relatively rapid attachment of the trypanosome to the macrophage and, secondly, the endocytosis of the parasite (Pepys, 1976; Takayanagi et al, 1974; Stevens and Moulton, 1978; Shaw and Griffin, 1981). This is demonstrated by the experiment involving the poisoning of the MPS by silica dust. While different quantities of silica were equally effective in reducing the hepatic uptake of passively immunised mice, there was a consistent residual amount of ^{75}Se-activity in the liver of these animals. This hepatic uptake is presumed to reflect those trypanosomes adhering to the surface of the macrophages which are unable to engulf the parasite. The present findings are in agreement with earlier in vitro studies on the phagocytosis of trypanosomes by peritoneal macrophages (Takayanagi et al, 1974a) in which a close correlation was found between opsonising activity and agglutination titres of HIS (Takayanagi et al, 1974). Furthermore, the ability of unlabelled trypanosomes to reduce not only the hepatic uptake of radiolabelled parasites but also the agglutination titre and the ability of HIS to restore hepatic uptake suggests that the limiting factor in trypanosome removal may not be the number of phagocytic cells available but the levels of circulating antibody. More recently Ferrante and Jenkin (1978) have also shown that T. lewisi maintained in diffusion chambers in the peritoneal cavity of immune rats were not lysed but strongly agglutinated at the end of the experiment. In addition, Stevens and Moulton/

Moulton (1978) have shown by electron microscopy studies that whole trypanosomes were phagocytosed and that they were digested and lysed within the macrophage.

It is also of interest that studies of T. musculi infections in C5-deficient, C3-depleted and normal mice showed that C3 depletion prolonged the infection whilst C5 deficient mice responded in a similar manner to normal mice (Jarvinen and Dalmasso, 1977). As a result of these studies, they concluded that complement mediated lysis was not involved in the control of T. musculi infections, and suggested that a complement mediated opsonisation was probably responsible for the elimination. In contrast, with the closely related parasite T. lewisi, the course of infection did not alter in C3-depleted or C5-deficient mice suggesting that complement was not essential for the elimination of the circulating parasite (Jarvinen and Dalmasso, 1976). Similar findings have been reported for T. brucei infections in chronically C3-depleted mice (Shirazi, Holman, Hudson, Klaus and Terry, 1980). Shirazi et al (1980) found no alteration in the pattern and the course of the infection and have suggested that IgM antibodies alone are probably adequate to control bloodstream infections.

In conclusion, the results presented in this chapter show that the removal of T. brucei from the circulation of immunised mice is largely accomplished by antibody mediated hepatic uptake. This is dependent on C3 at low anti-trypanosome antibody titres. No evidence was found to suggest that lysis is a prerequisite to macrophage uptake or that trypanosome sensitised or non-specific activation of macrophages were involved in immune clearance.

Chapter 3

STUDIES IN ANIMALS WITH ACUTE INFECTIONS

Introduction.

Although African trypanosome infections of man and domestic animals are characteristically sub-acute or chronic, acute fulminating infections are not uncommonly observed (Apted, 1970; Fiennes, 1970). The pathogenesis of such infections has not been fully evaluated but may depend upon a number of factors such as the strain of parasite, the size of the inoculum, the rate of replication (McNeillage and Herbert, 1968; Soltys and Woo, 1969), the degree of biochemical derangement (Moon, Williams and Witherspoon, 1968) and the release of haemolytic toxins by the parasite (Chi, Webb, Lambert and Miescher, 1977). However, a principal factor in determining the course of trypanosome infections is probably the effectiveness of the host's immune response. This is thought to depend largely on antibody although there is also evidence that macrophages and complement may play important contributory roles (see General Introduction and Chapter 3). In addition the generalised immunosuppression to heterologous antigens observed during trypanosome infections (Goodwin, Green, Guy and Voller, 1972; Murray, Murray, Jennings, and Urquhart, 1973; Freeman, Hudson, Longstaffe and Terry, 1973; Hudson et al, 1976) may play a crucial role in determining the pathogenesis of the infection.

If immunosuppression affects the response to trypanosome antigen then the pattern and course of infection may be due to the degree of parasite induced immunosuppression and the rapidity of its onset. Thus Hudson et al (1976) proposed that the initial parasitaemia totally suppressed the host's immune response and following a period of remission the second parasitaemia was uncontrolled and fatal. If this were the case in all infections, then neither acute nor chronic infections would occur. The/

The effectiveness of the immune response in acute infections may also be reduced by other factors. It is possible, for example, that the immunogenicity of rapidly replicating parasites may be reduced. Alternatively the rapid rate of parasite replication in such infections may essentially outpace antibody production.

This chapter outlines a series of experiments to investigate the immunological clearance of circulating trypanosomes during an acute fulminating infection. An acute infection which kills within 6 days of its injection into the host avoids many of the complications associated with antigenic variation and subsequent pathological changes during a relapsing infection. The rapidly fatal course observed in acute trypanosomiasis is discussed in terms of the humoral response to the parasite; the ability of the parasite to suppress the immune response of the host; and the importance of parasite replication rates. A comparison is also made with a stock of T. brucei which produces a relapsing infection following the initial parasitaemia.

Materials and Methods.

Parasites: A single stabilate of T. brucei derived from a stock of TREU 226 was used throughout the study except in two experiments where a stabilate of T. brucei derived from a stock of TREU 667 was used.

Experimental Animals: The experimental animals used were female CFLP mice and female Hooded Lister rats.

Irradiation of Experimental Animals: When necessary, mice and rats were sub-lethally irradiated 1 day prior to infection with 650 rad in a ^{60}Co source.

Radiolabelling/

Radiolabelling Techniques: ^{75}Se-labelled trypanosomes were prepared using the *in vivo* labelling technique described in Chapter 1. The trypanosomes were separated from infected blood by DEAE cellulose chromatography. The suspension of washed ^{75}Se-labelled trypanosomes was diluted with PBGS to give an injection dose of 1 x 10^8 organisms (approximately 4000 c.p.m.) per mouse.

One hour after the i.v. injection of labelled parasites the mice were killed and the distribution of radiolabelled parasites in spleen, liver and blood were determined.

Measurement of replication times of two stabilates of T. brucei: The replication time of *T. brucei* 226 or *T. brucei* 667 was measured in both irradiated and non-irradiated mice following infection with 1 x 10^4 trypanosomes. The parasitaemia was monitored twice daily, morning and afternoon, by examination of wet tail blood smears and quantified by the rapid matching method of Herbert and Lumsden (1976). The parasitaemic profile was plotted and the doubling time calculated by regression analysis.

Preparation of Hyperimmune serum: HIS was obtained from rats infected with *T. brucei* 226 as previously described (General Materials and Methods).

Vaccination of mice: Washed *T. brucei* 226 and *T. brucei* 667 were prepared using DEAE cellulose chromatography. The trypanosomes, suspended in PBGS and surrounded by ice, were irradiated with 60 krad.

Groups of mice were then vaccinated with either *T. brucei* 226 or *T. brucei* 667 by i.v. injection of 2 x 10^8 irradiated trypanosomes. Some of these groups were simultaneously infected with the stabilate not used for their vaccination.

IgM Plaque-forming Cell Assay (PFC): Mice were primed with 1 x 10^8 sheep erythrocytes (SRBC) and the direct (IgM) anti-SRBC response assayed 5 days later by a modification of the Jerne technique (Cunningham

and/

and Szenberg, 1968). The assay was performed on pooled spleen cells obtained from groups of 4 mice. Each test well contained 150 µl of a spleen cell suspension, 10 µl GPS and 20 µl of a 15% suspension of GPS adsorbed SRBC.

In vitro treatment of trypanosomes with serum:

(a) Neutralising antibody infectivity tests were performed on sera, as previously described (General Materials and Methods);

(b) Clearance studies were conducted following the in vitro treatment of ^{75}Se-labelled trypanosomes with serum, as described in Chapter 2. The ^{75}Se-labelled trypanosomes were then washed three times in PBGS to remove unbound HIS.

Results.

Clearance of ^{75}Se-labelled T. brucei by acutely infected mice:

In this experiment the ability of highly parasitaemic mice, first infected 4 days previously, to remove labelled trypanosomes was investigated. The results presented in Table 3.1 show that mice with a high parasitaemia do not differ appreciably from uninfected control mice. In these animals the majority of the injected activity remained in their blood. These results suggest that acutely infected mice had inadequate levels of opsonic antibody or an impaired phagocytic function.

The absence of effective levels of antibody was confirmed by infectivity neutralisation tests. In such tests pooled sera from infected and normal mice were both shown not to possess protective antibodies (100% mortality in both groups of five mice).

The effect of passive immunisation on the clearance of labelled trypanosomes by acutely infected mice:

In order to investigate if the failure of mice infected with T. brucei 226 to remove radiolabelled trypanosomes from their circulation was

due/

TABLE 3.1. The tissue distribution of radiolabelled homologous trypanosomes injected 1 h. previously into mice with a fulminating parasitaemia of T. brucei 226.

	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
Infected mice	5.5 ± 0.5	14.1 ± 0.6	69.8 ± 3.7
Uninfected mice	3.0 ± 0.4	12.4 ± 0.6	83.8 ± 4.8

TABLE 3.2. The effect of passive immunisation on the tissue distribution of ^{75}Se-labelled T. brucei in mice with a fulminating parasitaemia.

Group	Hyperimmune serum (ml)	Tissue distribution (% injected activity)		
		Spleen	Liver	Blood
Infected	0	4.4 ± 0.6	12.4 ± 0.7	81.4 ± 4.4
Infected	0.2	6.1 ± 0.9	15.1 ± 1.6	73.6 ± 3.3
Uninfected	0	<3.0	10.2 ± 0.8	83.9 ± 5.3
Uninfected	0.2	<3.0	62.1 ± 3.5	12.4 ± 2.1

due primarily to an absence of antibody, a group of trypanosome-infected mice was given an i.v. injection of HIS.

Since previous studies have demonstrated the efficacy of passive immunisation with relatively small amounts of HIS (0.01 ml) in promoting high hepatic uptake in normal mice (see Chapter 2), a single i.v. injection of HIS (0.2 ml) was administered to mice infected, 4 days previously, 15 mins. after the injection of radiolabelled trypanosomes.

The results presented in Table 3.2 show that this amount of HIS failed to increase hepatic uptake in acutely infected mice, i.e. approximately 15%, yet in passively immunised uninfected animals a high hepatic uptake of approximately 62% was obtained. The results show that infected animals again produced a hepatic uptake similar to uninfected controls, approximately 12% compared to 10%.

The low hepatic uptake found in passively immunised infected animals may be explained if the quantity of HIS was insufficient to effectively opsonise the large numbers of circulating parasites.

Alternatively, the failure of infected animals to achieve a high hepatic uptake may be a result of the saturation of the available phagocytes by the heavy parasitaemia.

The functional integrity of the MPS in highly parasitaemic mice:

In an attempt to assess the availability of hepatic macrophages for trypanosome removal in highly parasitaemic mice, infected 4 days previously, groups of mice received labelled T. brucei 226 which had been incubated in HIS prior to their injection. The results presented in Table 3.3 show that pre-treatment of the labelled trypanosomes enabled the parasitaemic mice to remove a large proportion of the labelled parasites from their circulation by hepatic uptake, i.e. approximately 62% compared to 15% in infected mice receiving labelled trypanosomes which had not been pre-treated with HIS.

Thus/

TABLE 3.3. The tissue distribution of ^{75}Se-labelled homologous T. brucei previously incubated in hyperimmune serum before injection into highly parasitaemic mice.

Group	Opsonised trypanosomes	Tissue distribution (% injected activity)		
		Spleen	Liver	Blood
Infected	-	5.8 ± 0.8	14.7 ± 0.6	61.1 ± 14.2
Infected	+	<3.0	61.9 ± 2.7	8.7 ± 2.1
Uninfected	+	<3.0	70.9 ± 1.6	<3.0

TABLE 3.4. The tissue distribution of ^{75}Se-labelled homologous trypanosomes injected into groups of mice which had been infected with T. brucei 226 5 days previously and treated with Berenil before injection of parasites.

Group	Interval between Berenil treatment and injection of radiolabelled trypanosomes	Tissue distribution (% injected activity)		
		Spleen	Liver	Blood
Infected	5 hrs.	<3.0	10.8 ± 0.5	74.4 ± 0.2
	24 hrs.	<3.0	56.5 ± 1.6	6.0 ± 0.5
Uninfected	24 hrs.	<3.0	10.7 ± 0.5	80.4 ± 3.5

Thus, a high parasitaemia per se does not impair the ability of macrophages to remove trypanosomes provided they are suitably opsonised.

The results of these three experiments indicate that the inability of infected animals to remove trypanosomes from their circulation is due to an apparent failure to achieve adequate levels of circulating antibody. This may be either a result of impaired antibody production associated with trypanosome-induced immunosuppression or parasite replication "outpacing" antibody synthesis and thereby leading to a relative deficiency of antibody despite increased production.

The relationship between parasitaemia and circulating antibody:

The adsorption of antibody on to the surface of replicating trypanosomes may prevent the mice from achieving effective levels of circulating antibody. To investigate this possibility diminazene aceturate was administered to groups of infected mice 5 hrs. and 24 hrs. before the injection of labelled parasites. The trypanocidal activity of this drug is thought to be due to its capacity to bind to kinetoplast DNA and thus prevent parasite replication (Newton and Le Page, 1967; 1968). The results are presented in Table 3.4.

Infected mice which had received Berenil treatment 24 hrs. before the injection of radiolabelled trypanosomes had the ability to remove a large proportion of the radiolabelled parasites from their circulation. In contrast, mice treated only 5 hrs. before the injection of labelled trypanosomes were incapable of such removal, approximately 56% compared to 11% respectively.

The examination of wet blood smears of infected blood 5 hrs. post-trypanocidal therapy showed the presence of numerous parasites; however by 24 hrs. there was a dramatic reduction; the parasitaemia falling from 10^9 trypanosomes per ml. of blood to less than 10^6 trypanosomes per ml. of blood.

The/

The results might suggest that in rapidly replicating infections of trypanosomes, antibody is being produced but not in sufficient quantities to promote obvious opsonisation and hepatic uptake. However, if replication is stopped even although circulating parasites can be detected, effective levels of antibody are achieved within 24 hrs.

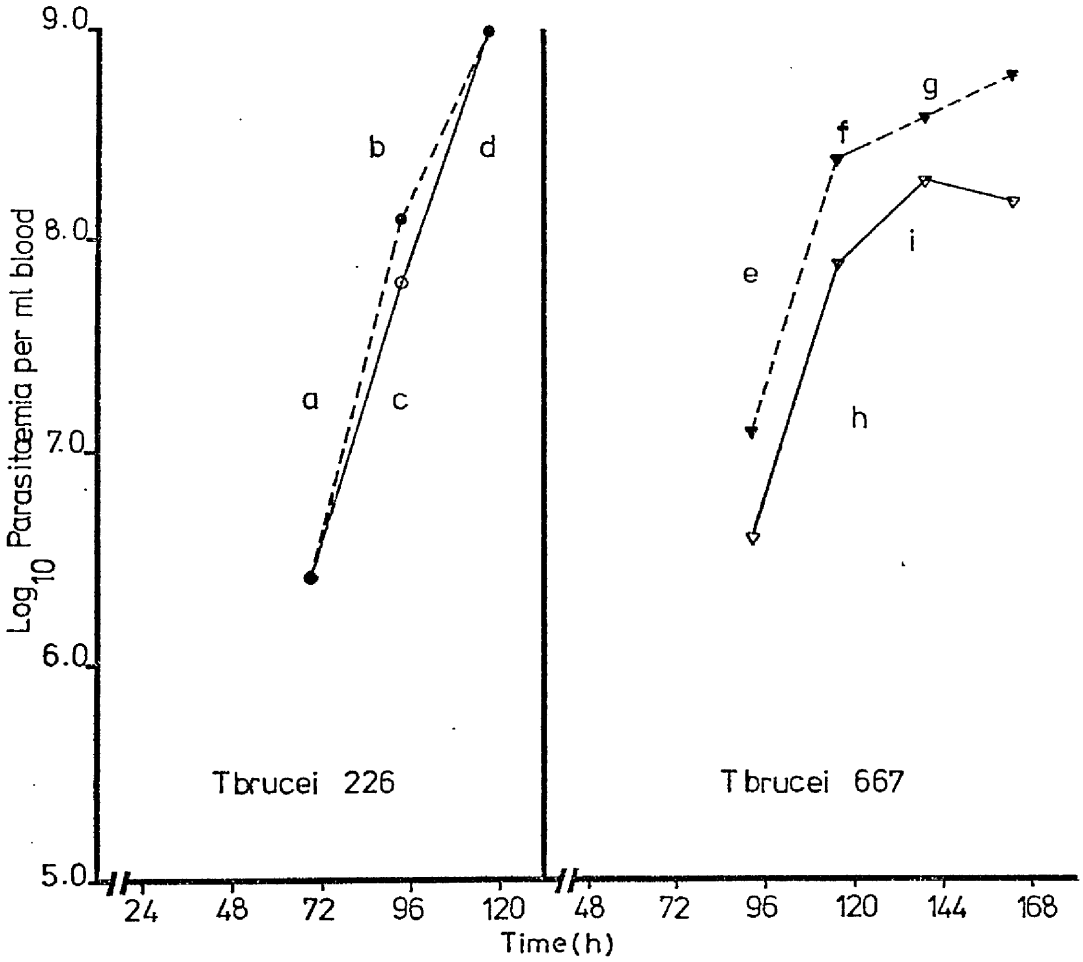
Parasite replication rates of two stocks of T. brucei in normal and irradiated mice:

The experiments described so far suggest that mice with virulent infections of T. brucei cannot achieve effective levels of circulating antibody because of the rapidly increasing parasitaemia. To evaluate the replication rate of T. brucei 226 its parasitaemic profile was compared with another stock of T. brucei 667 which causes a more chronic infection.

The rise in blood parasitaemia is presented in Fig. 3.1 and is based on four groups of six mice and shows that, whilst T. brucei 226 infection has a sustained single phase rate of growth, T. brucei 667 was characterised by a biphasic growth rate. During the initial period of growth, both strains had similar replication rates of approximately 5.5 hrs. However, during the critical period between 5 and 7 days post-infection, rates of replication were markedly different. In the acute infection (T. brucei 226) the replication rate remained constant and both groups of mice died by day 5. In contrast, the chronic infection (T. brucei 667) had a replication rate which slowed down markedly between day 5 and 7 in both irradiated and non-irradiated mice to 37 and 18 hrs. respectively.

Antibody was apparently essential for the control of the T. brucei 667 infection as irradiated animals died between days 7 and 8. It is also noteworthy that the parasitaemias were consistently higher in irradiated mice than in non-irradiated animals infected with T. brucei 667. Furthermore the maximum number of parasites attained in the circulation of infected mice was higher in T. brucei 226 infections; the animals dying with/

Figure 3.1 Parasitaemic profiles of T brucei 226 and 667 in normal (—) and irradiated (---) mice. Replication rates obtained by regression analysis are (a) 4h, (b) 7,9 h, (c) 5,2 h, (d) 5,9h, (e) 5,9h, (f) 37,2h, (g) 36,3h, (h) 5,4h and (i) 18,4h.



with blood parasitaemias in excess of 10^9 trypanosomes per ml. In contrast, the peak of parasitaemia in T. brucei 667 infections was approximately 3 x 10^8 trypanosomes per ml. in non-irradiated mice.

Clearance of ^{75}Se-labelled T. brucei 667 by chronically infected mice: The previous experiments suggest that a continued high rate of parasite replication may be an important factor in determining the inability of infected mice to remove trypanosomes from the circulation by hepatic uptake. The following experiment was therefore undertaken. Groups of mice were infected with T. brucei 667 13 days, 9 days, 6 days, 5 days and 4 days before the injection of ^{75}Se-labelled T. brucei 667.

The results presented in Table 3.5 show that during the phase of rapid growth between days 4 and 6 the mice were incapable of removing labelled parasites from the circulation, although there is a rise in the hepatic uptake by days 5 and 6 compared to controls, i.e. approximately 18% compared to 10%. However, mice infected for 9 days or 13 days were capable of removing large numbers of parasites by hepatic uptake approximately 46% compared to 12% in control mice.

IgM PFC response to SRBC in parasitaemic mice primed on the day of infection: Since it has been suggested that a direct relationship exists between the virulence of different strains of trypanosomes and their capacity to suppress IgM responses (Sacks, Selkirk, Ogilvie and Askonas, 1980), it is possible that our failure to detect circulating antibody in acutely infected mice and the rapid appearance of antibody following trypanocidal therapy may be due to the immunosuppressive effects of large numbers of living trypanosomes. To investigate this, direct (IgM) PFC assays were performed on mice which had been both infected with T. brucei 226 and primed with SRBC 5 days previously.

The results presented in Table 3.6 show that IgM antibody production to the SRBC at 5 days post-infection was as high as those of uninfected/

TABLE 3.5. The tissue distribution of ^{75}Se-labelled T. brucei 667 in T. brucei 667 infected CFLP mice.

	% injected activity		
	Spleen	Liver	Blood
13 day infection	3.8 ± 0.5	49.7 ± 6.1	< 3.0
9 day infection	14.4 ± 0.9	42.7 ± 11.9	< 3.0
6 day infection	5.7 ± 1.1	17.6 ± 1.8	39.1 ± 6.5
5 day infection	11.6 ± 0.4	18.1 ± 3.4	45.1 ± 3.6
4 day infection	9.1 ± 1.3	12.1 ± 0.7	55.7 ± 6.7
Control	8.6 ± 1.8	10.1 ± 0.9	46.5 ± 2.4

TABLE 3.6. The PFC response of acutely infected mice immunised with 1 x 10^8 SRBC on the day of infection and measured 5 days later.

Group	SRBC given day of infection	Anti-SRBC PFC per spleen*
Infected	+	77,388 ± 968
	-	< 500
Uninfected	+	71,398 ± 4,609
	-	< 500

* Average of 6 chambers.

uninfected controls. There was, therefore, no evidence to suggest that IgM antibody synthesis was impaired in acutely infected mice to an antigen presented contemporaneously with the trypanosome infection. A priori, it therefore seems probable that antibody production against the first parasitaemic peak would be similarly unimpaired.

Vaccination with irradiated organisms in mice simultaneously infected with trypanosomes of a different stock:

In order to evaluate directly anti-trypanosome antibody production in infected mice, groups of animals were vaccinated with irradiated trypanosomes of one stock of T. brucei on the same day as infection with non-irradiated organisms of another T. brucei stock. The effectiveness of the vaccination was then assessed 5 days later by the measurement of immune clearance of radiolabelled parasites of the same stabilate as that used for vaccination. The experimental design and results are presented in Table 3.7.

The results clearly show that neither infection with T. brucei 226 nor T. brucei 667 prevented successful vaccination against the other stabilate, as judged by the high hepatic uptakes, the hepatic uptake in all vaccinated groups being approximately 60%. An interesting feature of the ^{75}Se-T. brucei 667 clearances is that in T. brucei 226 infected mice an increased hepatic uptake occurs, approximately 30% compared to 15% in uninfected controls. It is not known whether this reflects a non-specific uptake of parasites by the liver due to the activation of hepatic macrophages, or to the formation of cross protective antibodies.

TABLE 3.7. The tissue distribution of ^{75}Se-labelled trypanosomes in mice vaccinated with irradiated trypanosomes of one stock and simultaneously infected with another stock.

Day 0		Day 5 tissue distribution (% injected activity)		
Vaccination	Infection	Spleen	Liver	Blood
		^{75}Se-*T. brucei* 226		
226	667	3.7 ± 0.1	60.2 ± 5.7	8.7 ± 2.8
226	-	3.4 ± 0.4	60.6 ± 6.1	6.4 ± 2.4
-	667	3.0 ± 0.1	10.6 ± 0.8	74.2 ± 3.2
-	-	< 3.0	12.4 ± 0.6	74.5 ± 1.9
		^{75}Se-*T. brucei* 667		
667	226	3.6 ± 0.6	64.8 ± 2.3	7.7 ± 1.6
667	-	< 3.0	61.4 ± 1.2	4.8 ± 1.0
-	226	8.2 ± 1.3	29.5 ± 2.9	65.3 ± 3.8
-	-	5.0 ± 1.3	15.2 ± 2.9	64.4 ± 2.1

Discussion.

Trypanosomiasis of man and animals is typically characterised by relapsing parasitaemias with antibody directed against each successive antigenic variant. However natural infections where death is associated with a massive parasitaemia some days after the first appearance of parasites in the blood are not uncommon (Chizyuka,1980). This chapter is concerned with an investigation of acute infections in an experimental model and, in particular, with the reasons underlying the apparent failure of the immune response.

Using the hepatic uptake of ^{75}Se-labelled trypanosomes as an index of the immune response (See chapters 1 and 2), we were unable to demonstrate the presence of any antibody-mediated uptake by the liver in mice 4 days after infection. The capacity of the MPS to remove parasites did not appear to be reduced by trypanosome blockade of the available phagocytes, as demonstrated by the large hepatic uptake in infected mice receiving trypanosomes pre-treated with HIS. The MPS is therefore still receptive to properly opsonised parasites, and thus one might expect that a larger percentage of the injected activity would have been removed in infected animals if adequate levels of circulating antibody were available. As demonstrated in Chapter 2, the injection of unlabelled trypanosomes into immune mice prior to clearance studies effectively reduces the hepatic uptake of ^{75}Se-labelled organisms which can be restored by passive immunisation using HIS. However passive immunisation with HIS failed to increase hepatic localisation of radio-labelled trypanosomes and suggests that large numbers of circulating parasites, 4 days after infection, reduced antibody to levels insufficient to achieve effective opsonisation and hence hepatic uptake. This possibility was supported by the results of an experiment in which acutely/

acutely parasitaemic mice were treated with the trypanocidal drug, Berenil, 24 hours before the injection of radiolabelled parasites. During this period the parasitaemia had fallen from 10^9 to 10^6 trypanosomes/ml. and after the injection of radiolabelled parasites their hepatic uptake was similar to that obtained in immune mice (see Chapter 2). Apparently, by preventing parasite replication, Berenil enables the host to overcome the static population of non-dividing trypanosomes.

A comparison of the replication rate of T. brucei 226 with that of T. brucei 667 which causes a chronic infection, showed that the virulent 226 infection had a constant replication rate of 5.7 hours between the time of first detection in the blood on day 3 and death on day 5. In the relapsing stock, 667, the replication rate between day 4, when the parasites were first detected in the blood, and day 5 was 5.4 hours but thereafter decreased to 18.4 hours, so that the peak parasitaemia of 667 occurred one day later and did not reach the level of the 226 infection.

Two aspects of the parasitaemic profile of 667 are of interest. First, the change in replication rate which occurred 120 hours after infection was also observed in sub-lethally irradiated mice indicating that it was not antibody-mediated but was an inherent characteristic of the strain and possibly associated with the development of intermediate and stumpy forms which occur towards the peak of parasitaemia in relapsing strains (Hoare, 1970; Balber, 1972). The second aspect is the actual decrease in circulating parasites which occurred after day 6. This could be reasonably attributed to the appearance of adequate amounts of antibody since it did not happen in irradiated mice whose parasitaemia continued to increase until death one or two days later. Indeed T. brucei infected/

infected mice which have controlled the initial parasitaemia are able to remove radiolabelled parasites of the original variant at various stages after the remission. This finding, together with the inability of T. brucei 226 infected mice to remove radiolabelled parasites, suggests that the acute nature of the 226 infection results from the failure of the host to achieve effective levels of circulating antibody. Balber (1972) found that a relapsing T. brucei infection produced an increase in the protective antibody levels over the course of the infection. In contrast, it has been reported that gamma-globulins fall during an acute infection (Hara, Oka, Takagi, Nagata and Sawanda, 1955). Thus there is an inverse relationship between the numbers of circulating dividing parasites and the ability of the host to control the initial parasitaemia.

Recently Sacks et al (1980) have proposed that the virulence of different strains of trypanosomes, as measured by survival times, is directly correlated with their ability to induce immunosuppression. The latter was determined on the basis of altered PFC responses to SRBC in mice inoculated with membrane fractions of different strains of trypanosomes. While it is accepted tnat immunosuppression may be an important factor in survival of animals with sub-acute or chronic infections, there was no evidence that it occurred in this particular acute infection. Thus mice inoculated with SRBC at the time of infection with 226 produced PFC responses comparable to those of uninfected mice. The failure to detect parasite induced immunosuppression in this acute infection was confirmed when mice simultaneously vaccinated with irradiated trypanosomes of one stock of T. brucei and infected with another stock, developed by day 5 levels of opsonic antibody comparable to those of vaccinated controls. Neither of these results would be expected in immunosuppressed mice.

Hudson/

Hudson and Terry (1979) have shown that the residual IgM in chronically infected animals is sufficient to enable the host to control blood parasitaemia. If, however, the production of antibody (IgM) was totally suppressed by cyclophosphamide therapy, then the parasitaemia was uncontrolled.

It is therefore apparent that factors affecting the effectiveness of the available IgM may have dramatic consequences. Thus a fine balance exists between the level of circulating IgM and the rate at which it is removed from the circulation by the increasing parasite population.

It is concluded that the acute fatal infections of trypanosomes observed in these experiments were the result of the inability of the host to achieve effective levels of circulating antibody. This was not due to any significant degree of immunosuppression but rather to the continued rapid replication rate of such strains of trypanosomes which constantly outpaced antibody production. In contrast, the strain of trypanosome which causes a more chronic infection, although initially having a similar replication rate, subsequently switched to a slower one and thereby allowed antibody to reach levels which permitted effective opsonisation. Furthermore, it is interesting to note that the majority of acute natural trypanosome infections reported are a result of T. evansi, a monomorphic strain of trypanosome, whilst chronic infections are usually the result of pleomorphic trypanosome infections. Indeed Barry, le Ray and Herbert (1979) have suggested that the virulence of a particular strain of trypanosome is dependent on the degree of pleomorphism rather than the rate of replication.

It is also possible, in sub-acute and chronic infections in which immunosuppression to heterologous antigen is well established, that the replication rate of particular variants might be equally important/

important in determining the outcome of infection, as recently suggested by Hudson and Terry (1979).

SECTION II.

ASPECTS OF THE IMMUNE RESPONSE IN MICE AND CATTLE INFECTED WITH *T. CONGOLENSE*.

Chapter 4

GENETIC RESISTANCE OF MICE TO *TRYPANOSOMA CONGOLENSE*

Introduction.

It is now widely accepted that some breeds of cattle, particularly those of West Africa, are less susceptible to trypanosomiasis than the majority of cattle maintained in the tsetse belt of tropical Africa (Stewart, 1951; Chandler, 1952). In the majority of experiments into this phenomenon of reduced susceptibility, the N'dama breed of cattle has invariably shown the greatest degree of trypanotolerance, whilst the Zebu type cattle breeds have shown the least. The basis of this trypanotolerance is unknown. However, trypanotolerant breeds are in general the ancient indigenous breeds of West Africa. In contrast, the more recently introduced Asiatic Zebu type cattle breeds tend to be more susceptible to the effects of the disease. Consequently, trypanotolerance has historically been presumed to have a genetic basis.

Trypanotolerance is probably a complex relationship which may be affected by the level of tsetse challenge, the nutritional status of the host and physical characteristics such as hide thickness. However, the main feature of trypanotolerant breeds is their ability to limit the numbers of circulating parasites and maintain their PCV (Fiennes, 1970). Holmes and Jennings (1976) have shown that the development of an anaemia in trypanosome infected animals is closely associated with the parasites in the circulation. Furthermore, Desowitz (1959) has suggested that trypanotolerant cattle produce a more effective immune response than susceptible breeds. Thus the ability to control the number of circulating parasites may be directly related to the efficacy of the immune response.

Recently Jennings and Whitelaw (1977) have described an experimental model of trypanotolerance using C57Bl mice. In this model of genetic resistance C57Bl mice are able to control the peripheral parasitaemia and produce a relapsing infection lasting beyond 100 days. In contrast, most strains, including CFLP mice, are susceptible to the infection/

infection, fail to control the initial parasitaemia and die within 20 days.

This chapter details a series of experiments in such mice designed to investigate the basis of trypanotolerance in terms of differences in the efficacy of the immune response to trypanosome infection.

Materials and Methods.

Parasite: A stabilate of T. congolense derived from a stock of T. congolense GVR 1 was used in all experiments. In addition, a stabilate of T. brucei derived from a stock of T. brucei TREU 226 was used in one experiment.

Experimental animals: Female CFLP mice, C57Bl mice and female Hooded Lister rats were used.

Infection: Mice and rats received 1 x 10^4 and 1 x 10^5 trypanosomes suspended in PBGS by i.p.injection respectively. The parasitaemia was estimated by examining wet tail blood smears using the rapid matching method of Lumsden and Herbert (1976).

Radiolabelling techniques: Radiolabelled parasites were prepared by the in vivo method using ^{75}Se -methionine previously described in Chapter 1. The tissue distribution of radiolabelled parasites was determined by the method previously described (Chapter 1). In addition, the radioactivity per gram of tissue was expressed as a percentage of the injected activity because hepato - splenomegaly commonly occurs during T. congolense infections.

Immunoglobulin estimation: Serum levels of IgM and IgG were quantified from the day of infection by radial immunodiffusion (Mancini, Carbonara and Heremans, 1965) using antisera against mouse IgM and mouse IgG prepared in rabbits (Nordic Immunochemical Reagents Ltd., Berks.).

Infectivity/

Infectivity neutralisation tests: These were performed as described previously in the General Materials and Methods.

Irradiation of experimental animals: When necessary experimental animals were sub-lethally irradiated with 650 rad in a ^{60}Co source one day prior to their infection.

Vaccination of mice: An irradiated T. brucei 226 vaccine was prepared as described in Chapter 3.

Activation of MPS: All immunostimulants were given by a single i.v. injection on the day of infection unless otherwise atated.

(i) Corynebacterium parvum. Each mouse received 0.2 ml. of a suspension of C. parvum (Coparvax, Wellcome Ltd., Kent) approximately 1.4 mg. dry weight of organisms. In addition one group of mice received a similar dose by i.p. injection.

(ii) Freunds complete adjuvant. 0.2 ml. of Freunds complete adjuvant (Difco Laboratories, U.S.A.) was given as an i.p. injection.

(iii) Levamisole Hydrochloride. Each mouse received 75µg levamisole (Nemicide, I.C.I. Ltd., Cheshire) by subcutaneous injection(s.c.).

(iv) Escherichia coli lipopolysaccharide. Mice were given 10 µg of E. coli LPS (Sigma Chemical Co. Ltd., London).

(v) Bordetella pertussis. B. pertussis was kindly donated by Professor A.C. Wardlaw of the Department of Microbiology. Each mouse received 5×10^9 organisms in 0.2 ml.

(vi) Bacillus Calmette-Guerin. B.C.G. vaccine (Glaxo Laboratories, Greenford) containing 1.5×10^7 Mycobacterium bovis organisms was inoculated into each mouse.

Preparation/

Preparation of hyperimmune serum. Hyperimmune serum against T. congolense GVR 1 was prepared in CFLP mice by infection, followed by Berenil treatment after seven days. The mice subsequently received two challenge infections seven and twenty-one days later. Serum was collected five days after the second challenge and pooled.

Trypanolysis of ^{75}Se-T. congolense. ^{75}Se-labelled T. congolense 1×10^8 organisms in 0.1 ml. were incubated with serum (0.2 ml) from C57Bl or CFLP mice together with GPS (0.2 ml) for 2 hrs. at 37°C. To achieve maximum release of ^{75}Se-activity 0.2 ml of NP40(10%) or deoxycholate (20%) was used in the place of test serum. Minimum ^{75}Se-release was assessed from ^{75}Se-radiolabelled parasites incubated in PBGS or NMS. Each test was carried out in triplicate.

After incubation 1 ml. of PBGS was added to each tube, thoroughly mixed, spun for 30 mins. (650g.) at 4°C. and 0.5 ml. of supernate pipetted into a separate tube. The percentage lysis was calculated as

$$\% \text{ lysis} = \frac{\text{Supernate c.p.m. x 3}}{\text{Total c.p.m. of pellet + supernate}} \times 100$$

Results.

Pattern and course of T. congolense infection in C57Bl and CFLP mice: It has previously been reported (Jennings and Whitelaw, 1977) that C57Bl mice are less susceptible to T. congolense than the majority of other strains. Thus C57Bl mice can survive T. congolense infection beyond 100 days despite a continuous relapsing parasitaemia. In contrast, CFLP mice are highly susceptible to the infection and die within 20 days with the majority dying by day 12, without any reduction in the initial high parasitaemia.

In/

In order to confirm and extend these findings, groups of C57Bl and CFLP mice were infected with T. congolense GVR 1 and the development of the infection monitored twice daily. The results presented in Fig. 4.1. show that C57Bl mice are able to control and reduce the initial parasitaemia while CFLP mice fail to reduce the high peripheral parasitaemia and die by day 10. In addition, the peak parasitaemia is slightly lower in C57Bl mice when compared to CFLP mice and, although the initial growth in the peripheral parasitaemia is similar in the two strains, from day five onwards the rate of growth is apparently slower in C57Bl mice.

These findings were further investigated on the basis of differences in the immune response to trypanosomiasis in the two strains of mice.

The pattern and course of T. congolense infection in the absence of antibody:

To investigate the role of the immune response in the control of the peripheral parasitaemia in C57Bl mice, the following experiment was undertaken.

A group of C57Bl mice was immunosuppressed by sub-lethal irradiation and together with a group of non-irradiated C57Bl mice infected with T. congolense. The blood was monitored daily for the appearance of parasites.

The results illustrated in Fig. 4.2 show that irradiated C57Bl mice are unable to reduce and finally control the initial parasitaemia. Thus, the ability of C57Bl mice to control and survive an infection would appear to be influenced by the presence of an intact immune response.

In/

Figure 4.1

Parasitaemic profiles of C57Bl (▽—▽) and CFLP (● -●) mice infected with Trypanosoma congolense (GVR 1)

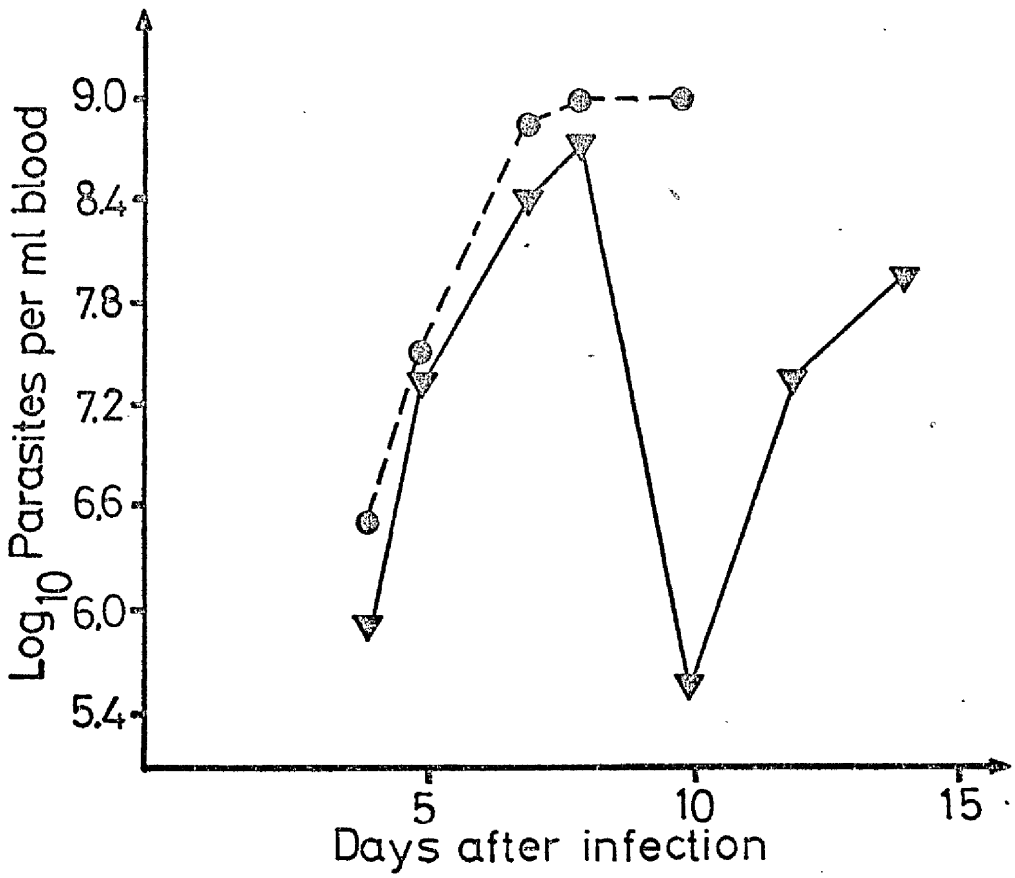
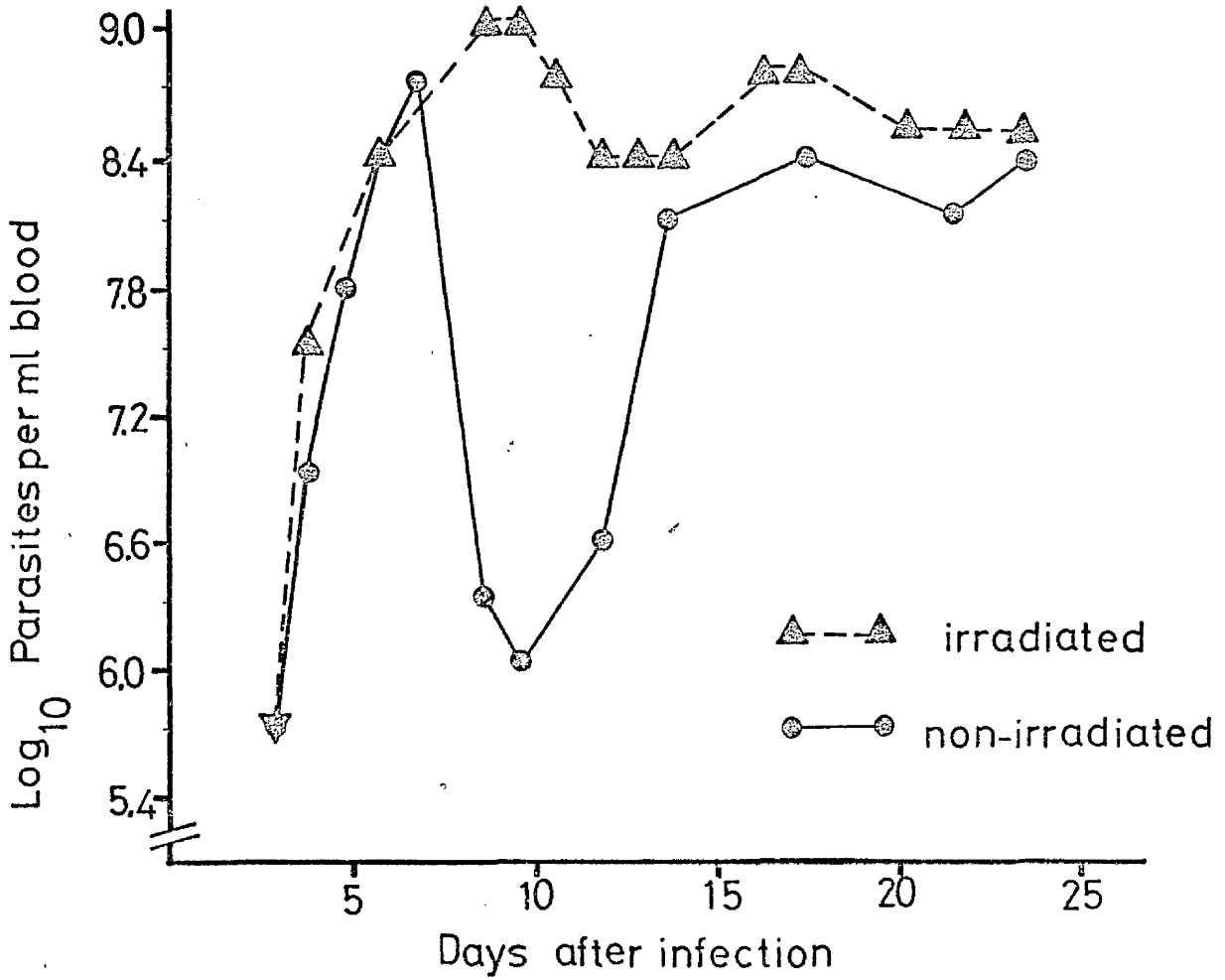


Figure 4.2 Effect of irradiation on the pattern and course of T.congolense in C 57Bl mice



In addition, the growth in peripheral parasitaemia slows down at a similar rate in both irradiated and non-irradiated mice until day 5. The parasitaemia is then rapidly reduced in non-irradiated mice while 4 out of 5 irradiated animals are dead by day 12.

Clearance of ^{75}Se-labelled T. congolense by infected C57Bl and CFLP mice:

The successful use of ^{75}Se-T. brucei in assessing the immune response in mice has been previously described (Chapters 1, 2 and 3). In this experiment ^{75}Se-labelled T. congolense were used to assess the immune response of C57Bl and CFLP mice to trypanosome infection. Groups of both strains had been infected 9 days prior to the injection of labelled trypanosomes.

The results presented in Table 4.1 show that C57Bl mice have an increased hepatic uptake compared to non-infected C57Bl mice, i.e. approximately 45% compared to 7% whilst infected CFLP mice have hepatic uptakes of approximately 20% compared to non-infected CFLP mice of 9%. The difference in hepatic uptake observed in CFLP mice can be accounted for by the increase in liver weight. However, this explanation does not wholly account for the increased hepatic uptake in infected C57Bl mice.

Furthermore, when radiolabelled T. congolense are injected into C57Bl mice infected for 42 days, 87 days and 100 days, the hepatic uptake is greater than non-infected C57Bl mice (Table 4.2). Thus, on average, mice infected for 42 days or more are able to remove from their blood approximately 40% of the injected radiolabelled parasites compared to the hepatic uptake of non-infected C57Bl mice of approximately 7%.

Clearance of ^{75}Se-labelled T. congolense by CFLP infected mice following Berenil chemotherapy:

It has previously been shown that Berenil chemotherapy rapidly increases the ability of T. brucei infected mice to remove ^{75}Se-labelled parasites from their circulation (Chapter 3). This response was now investigated in T. congolense infected animals. Two groups of 5 CFLP mice were infected/

TABLE 4.1. Tissue distribution of ^{75}Se-labelled T. congolense in C57Bl and CFLP mice infected 9 days previously.

Group	Tissue distribution (% of the injected activity)	
	Spleen	Liver
Infected C57Bl	9.4 ± 0.7 (17.0 ± 1.9)*	45.2 ± 1.9 (22.5 ± 2.5)
Infected CFLP	8.0 ± 1.4 (12.0 ± 1.6)	20.7 ± 1.2 (8.6 ± 0.5)
Control C57Bl	< 3.0 (23.2 ± 2.2)	7.5 ± 0.1 (5.2 ± 0.3)
Control CFLP	< 3.0 (29.4 ± 3.8)	9.5 ± 0.6 (7.6 ± 0.4)

TABLE 4.2. Tissue distribution of ^{75}Se-labelled T. congolense in chronically infected C57Bl mice.

Group	Tissue distribution (% injected activity)	
	Spleen	Liver
100 day	15.8 ± 5.2 (6.5 ± 1.3)	38.2 ± 4.3 (12.3 ± 0.3)
87 day	10.8 ± 2.3 (6.8 ± 1.8)	37.8 ± 5.0 (13.0 ± 2.0)
42 day	11.6 ± 1.8 (4.8 ± 0.5)	45.6 ± 1.9 (12.3 ± 0.6)
Control	< 3.0 (23.2 ± 2.2)	7.5 ± 0.1 (5.2 ± 0.3)

* values in parenthesis are the tissue distribution of radiolabelled parasites calculated from the c.p.m./gram of tissue expressed as a % of the injected activity.

infected and 24 hrs. prior to the injection of ^{75}Se-labelled parasites and 8 days after infection one group of mice was treated with Berenil.

The results (Table 4.3) show that chemotherapy does increase the ability of infected mice to remove parasites by hepatic uptake, approximately 57% in mice receiving Berenil compared to 18% in infected mice not receiving Berenil. This hepatic uptake compares well with that of immune mice of approximately 61%.

Infectivity neutralisation tests: The pooled serum obtained from 7 & 8 days and 9 & 10 days after infection from both C57Bl and CFLP mice were tested for the presence of neutralising antibody. The results indicated that serum from infected C57Bl mice was protective. Thus 7/8 day serum protected 4 out of 5 recipient mice, while 9/10 day serum protected 5 out of 5 recipient CFLP mice. In contrast, the serum from infected CFLP collected over a similar period and serum from non-infected C57Bl mice and CFLP mice completely failed to confer protection and all groups of recipient mice died by day 12.

^{75}Se-labelled T. congolense trypanolysis: To further investigate the apparent superior immune response by C57Bl mice to T. congolense infection, sera from infected C57Bl mice and infected CFLP mice were incubated with ^{75}Se-labelled parasites in the presence of GPS The release of ^{75}Se-activity into the supernate was used as a measure of the trypanolytic ability of the respective sera.

The results presented in Table 4.4 show that serum from infected C57Bl mice consistently produced a greater release of ^{75}Se-activity from radiolabelled parasites than serum from infected CFLP mice.

Although serum from infected CFLP mice does produce a higher release of radiolabel than control sera, i.e. 22% compared to 15%, it never reaches the levels observed using infected C57Bl mice serum, i.e. approximately 53%.

The/

TABLE 4.3. The tissue distribution of ^{75}Se-labelled T. congolense by infected CFLP mice following chemotherapy.

Group	Tissue distribution (% injected activity)	
	Spleen	Liver
Infected treated CFLP	6.8 ± 1.3 (10.8 ± 1.6)*	57.5 ± 6.8 (25.8 ± 1.5)
Infected CFLP	7.2 ± 1.3 (11.3 ± 1.0)	18.3 ± 1.3 (9.0 ± 0.9)
Control CFLP	3.6 ± 0.3 (25.6 ± 2.5)	9.9 ± 0.6 (8.6 ± 1.3)
Immune CFLP	3.9 ± 0.2 (16.8 ± 2.0)	61.5 ± 2.3 (34.8 ± 0.7)

* values in parenthesis are the tissue distribution of radiolabelled parasites calculated from the c.p.m./g. of tissue expressed as a % of the injected activity.

TABLE 4.4 Trypanolysis of ^{75}Se-labelled T. congolense.

Group	% Lysis
Infected C57Bl (7/8 day)*	56.4 ± 1.8
Infected CFLP (7/8 day)	24.9 ± 1.1
Infected C57Bl (9/10 day)**	50.5 ± 1.6
Infected CFLP (9/10 day)	19.1 ± 2.0
10% NP 40	79.2 ± 1.7
20% deoxycholate	100
N.M.S.	14.8 ± 0.7
PBS	13.2

* pooled serum from mice killed on days 7 and 8 of an infection.

** pooled serum from mice killed on days 9 and 10 of an infection.

The decrease in trypanolytic action of pooled infected sera from days 7 and 8 to days 9 and 10 may reflect consumption of antibody in vivo by the high numbers of circulating parasites. Thus the immune response of C57Bl mice, at least in terms of the production of trypanolysins, is more efficient than that of CFLP mice.

The ability of C57Bl mice to survive an infection while CFLP mice succumb, presumably reflects the differences in the effectiveness of the immune response in either quantity or quality of antibody formation.

The immunoglobulin response of C57Bl mice and CFLP mice following infection with T. congolense:

The development of the immunoglobulin response in C57Bl and CFLP mice to infection was monitored from the day of infection by collecting daily serum samples over the first 12 days of infection. The levels of immunoglobulin were measured by radial diffusion (Mancini et al, 1965).

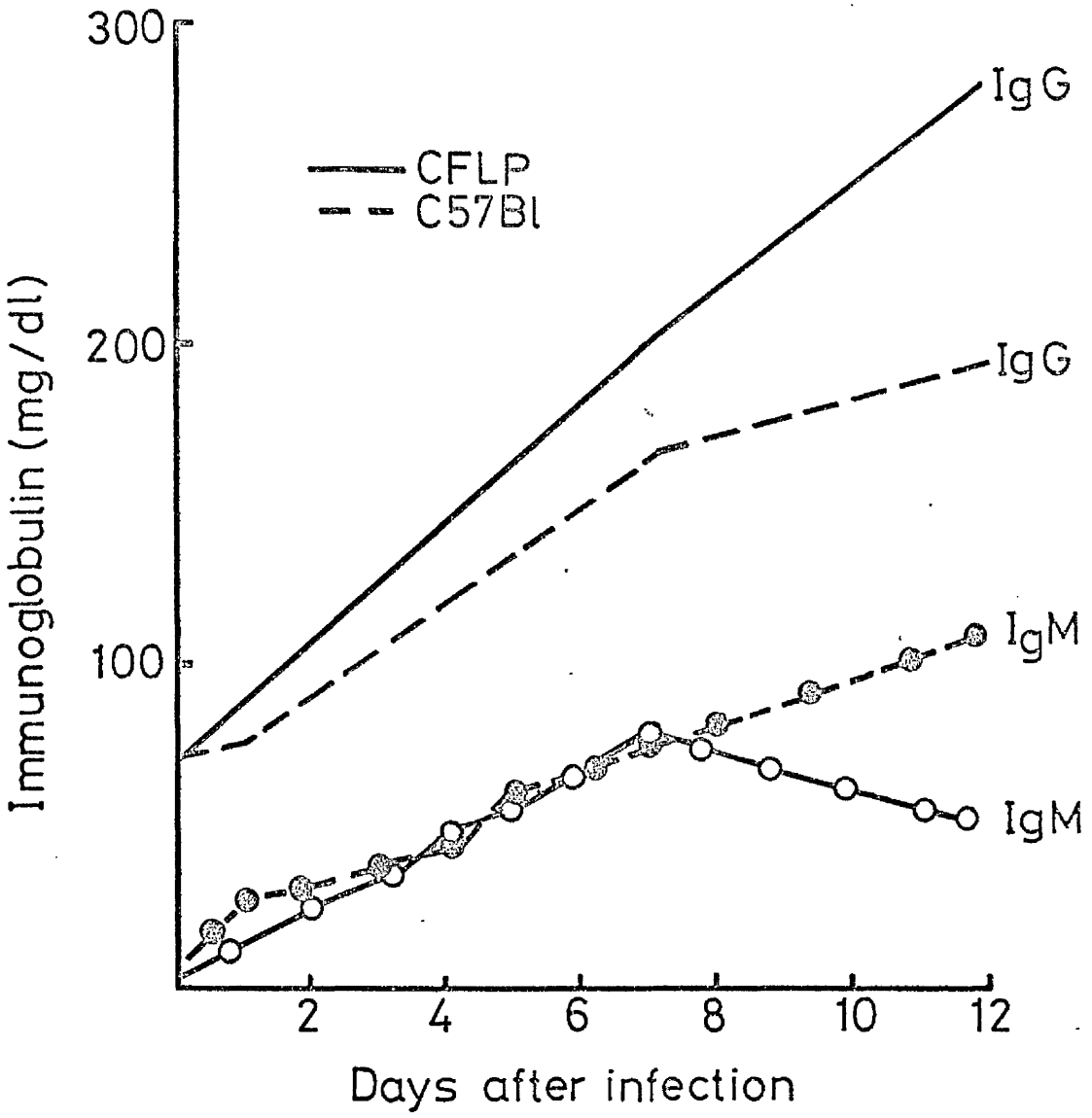
Figure 4.3 shows that both strains react to infection by increased production of IgM and IgG. In C57Bl mice IgM production is continuous over this period while in CFLP mice IgM production rises until day 7 at which point the level of IgM in the serum steadily falls. IgG production in CFLP mice is almost continuous throughout the period of infection, while in C57Bl mice the level of IgG production until day 7 is similar to CFLP mice. However, beyond this the rise in serum IgG slows.

Thus in trypanotolerant C57Bl mice the level of IgM in the serum remains high over the initial 12 day period, while in the susceptible CFLP mice the level of IgM begins to fall by day 7.

Plaque forming cell response to SRBC in infected C57Bl and CFLP mice:

Although it has been shown that immunosuppression is not an important factor in determining the outcome of acute T. brucei infections, the reduced/

Figure 4.3 The immunoglobulin response of C57Bl and CFLP mice to infection



reduced IgM formation in CFLP mice during T. congolense infection may be associated with a greater susceptibility of CFLP mice to parasite induced immunosuppression. Hudson et al (1976) have shown that high IgM levels are related to polyclonal activation of B cells resulting finally in immunosuppression due to depletion of antigen reactive B-lymphocytes. To investigate the possibility that the failure of CFLP mice to control the initial parasitaemia was as a result of a depressed immune response, the following experiment was undertaken. Groups of CFLP and C57Bl mice were infected with T. congolense and five days later some of these groups together with non-infected control mice received SRBC. After a further five days the direct PFC response of these mice was assessed, i.e. on day 10 of the infection. Table 4.5 shows that non-infected C57Bl mice produce fewer plaque forming cells per spleen than non-infected CFLP mice, i.e. 57,000 compared to 130,000. In contrast, infected C57Bl mice produce more plaque forming cells than infected CFLP mice (15,000 compared to 7,000). As direct PFC responses measure IgM, the increased PFC response of infected C57Bl mice can reasonably be associated with the increased serum IgM.

It also suggests that C57Bl mice are less severely affected by any immunosuppressive effect the parasite may have, at least to the T cell dependent antigen SRBC.

Vaccination of C57Bl mice chronically infected with T. congolense:

The previous experiment suggests that immunosuppression of antibody responses may be an important factor in determining the susceptibility of mice to T. congolense infection. However, C57Bl mice commonly produce chronic T. congolense infections of many weeks' duration. Thus the ability of these mice to continue to control each antigenic variant suggests that these animals are not totally immunosuppressed and are partially able to mount an effective immune response, at least against trypanosome/

TABLE 4.5 Plaque forming cell response of C57Bl and CFLP mice infected with T. congolense.

Day 0		Day 5	Day 10 *
C57Bl CFLP	T. congolense	SRBC	15,000 ± 278 7,000 ± 195
C57Bl CFLP	T. congolense	-	< 500 800 ± 80
C57Bl CFLP	-	SRBC	57,000 ± 605 130,000 ± 1,500
C57Bl CFLP	-	-	< 500 < 500

* values represent the average of three chambers.

TABLE 4.6 Tissue distribution of ^{75}Se-T. brucei 226 in C57Bl mice chronically infected with T. congolense 5 days after vaccination with irradiated T. brucei 226.

	Tissue distribution (% injected activity)	
	Spleen	Liver
Non-infected C57Bl vaccinated	6.1 ± 0.5 (24.0 ± 5.8)*	58.6 ± 5.2 (43.3 ± 6.4)
Non-infected CFLP vaccinated	5.5 ± 0.6 (14.7 ± 0.3)	60.6 ± 2.7 (26.0 ± 1.0)
Chronic C57Bl vaccinated	9.9 ± 2.3 (10.0 ± 3.5)	59.4 ± 5.7 (33.5 ± 2.5)
Non-infected CFLP non-vaccinated	9.1 ± 2.0 (38.0 ± 4.2)	13.4 ± 1.2 (6.0 ± 1.0)

* values in parenthesis are c.p.m./g. of tissue as % injected activity.

trypanosome antigen. To investigate this C57Bl mice chronically infected with T. congolense were vaccinated with irradiated T. brucei 226 on day 60 of the infection. The development of the immune response to this vaccination was assessed 5 days later when ^{75}Se-labelled T. brucei 226 were injected into these mice and the tissue distribution measured. The results are presented in Table 4.6 and show that the ability of chronically infected C57Bl mice to respond to trypanosome antigen is not as severely impaired as might be suggested from horse RBC experiments (Whitelaw, MacAskill, Holmes, Jennings & Urquhart, 1980).

This second series of experiments has shown that the ability of C57Bl mice to control the initial parasitaemia, and indeed possibly successive parasitaemias, resides in their ability to maintain effective levels of antibody, possibly IgM.

The last series of experiments was designed to investigate the possibility of enhancing the immune response of CFLP mice in an attempt to change their susceptibility to T. congolense infection.

Passive immunisation of CFLP mice infected with T. congolense:

Undoubtedly serum from infected C57Bl mice is highly active against trypanosomes as assessed by a variety of methods (vide supra) -

(a) In an attempt to confer protection on CFLP mice against infection, serum from C57Bl mice infected for 10 days was injected i.v. in 0.2 ml. aliquots into CFLP mice either on days +5, +6, +7 and +8; 0.5 ml. on day +5; or 0.5 ml. on day 9 of infection. None of these methods conferred any protection on CFLP mice and all died by day 12 of the infection.

(b) A second regime of passive immunisation was tried using hyperimmune serum obtained from CFLP mice. These hyperimmunised mice resisted infection when challenged with T. congolense and their serum was therefore/

therefore concluded to be protective. Groups of CFLP mice received 0.2 ml. aliquots of HIS on days -1, 0 and +1 or days +5, +6 and +7 of infection. The results obtained (Fig. 4.4) showed that again protection was not achieved and only when serum was given on days -1, 0 & +1 was the prepatent period extended.

To eliminate the possibility that the serum was being diluted out in the circulation an infectivity test using $^{1}/_{5}$ and $^{1}/_{10}$ dilutions of HIS was performed. In both cases 100% protection against infection was achieved in groups of 5 CFLP mice.

The effect of the number of infecting organisms on the subsequent parasitaemia:

In an attempt to allow the immune response of CFLP mice time to produce an effective response groups of CFLP mice were infected with 1×10^{1}, 1×10^{2}, 1×10^{3} or 1×10^{4} T. congolense. The appearance of parasites was monitored and is presented in Fig. 4.5. The reduction of infective doses from 1×10^{4} to 1×10^{1} produced an increase in the length of the prepatent period. There was also an indication that by reducing the infective dose, the actual time from first detection of the parasites in the bloodstream to death was increased. However, in none of the groups of mice was the initial parasitaemia controlled and indeed all mice eventually died by day 20.

Immunopotentiation of the mononuclear phagocytic system (MPS):

Various bacterial and chemical compounds have the effect of causing hypertrophy and hyperplasia of the MPS. Indeed some have been reported as conferring some degree of non-specific immunity against protozoan infections (Nussenzweig, 1967; Clark et al, 1976; Clark et al, 1977). The injection of various substances was therefore carried out in an attempt to enhance the activity of the MPS and thereby facilitate a reduction in the peripheral parasitaemia.

The/

Figure 4.4 The effect of passive immunisation on the pattern and course of T.congolense in CFLP mice

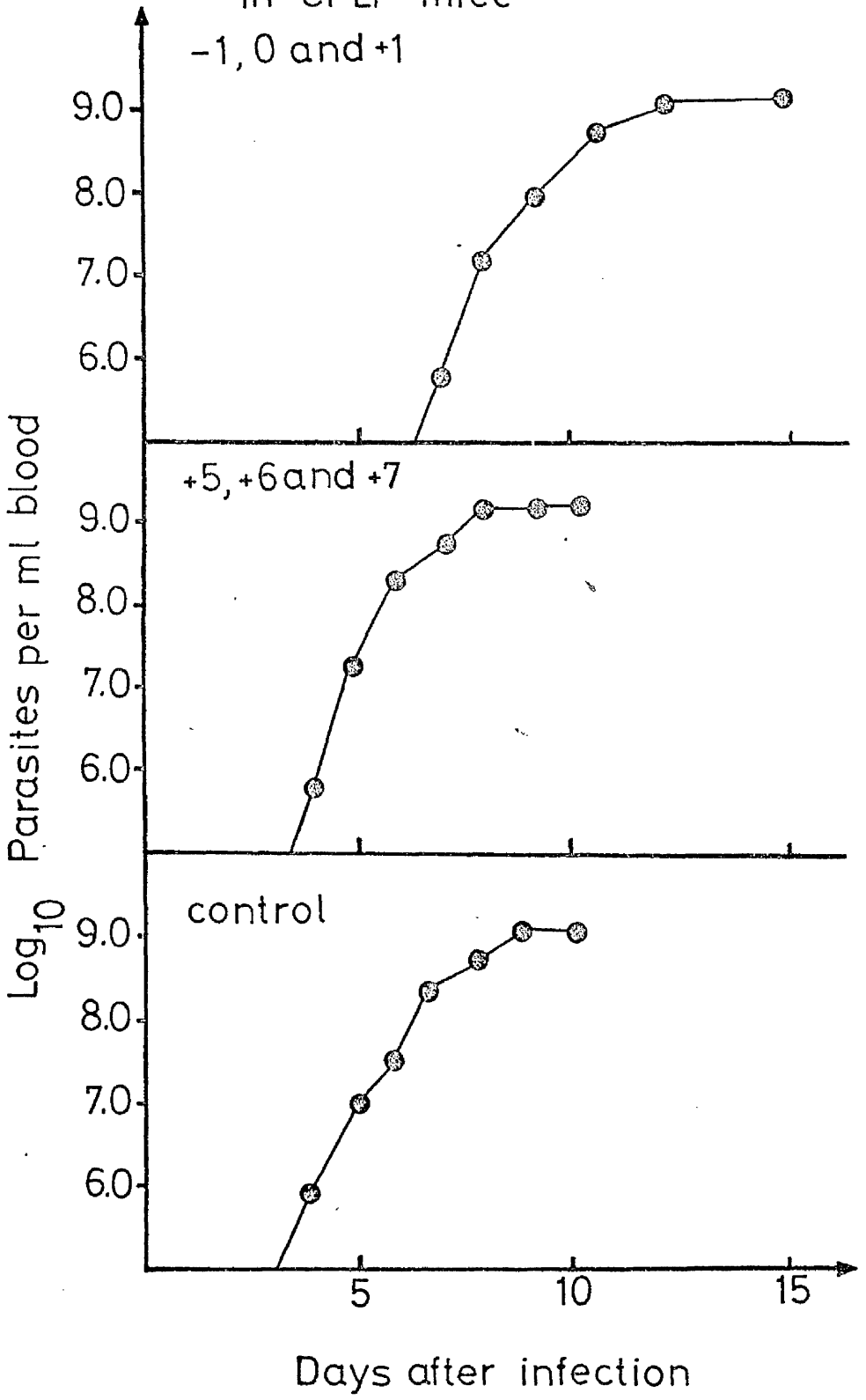


Figure 4.5 The influence of various doses of T. congolense on the pattern and course of the infection in CFLP mice

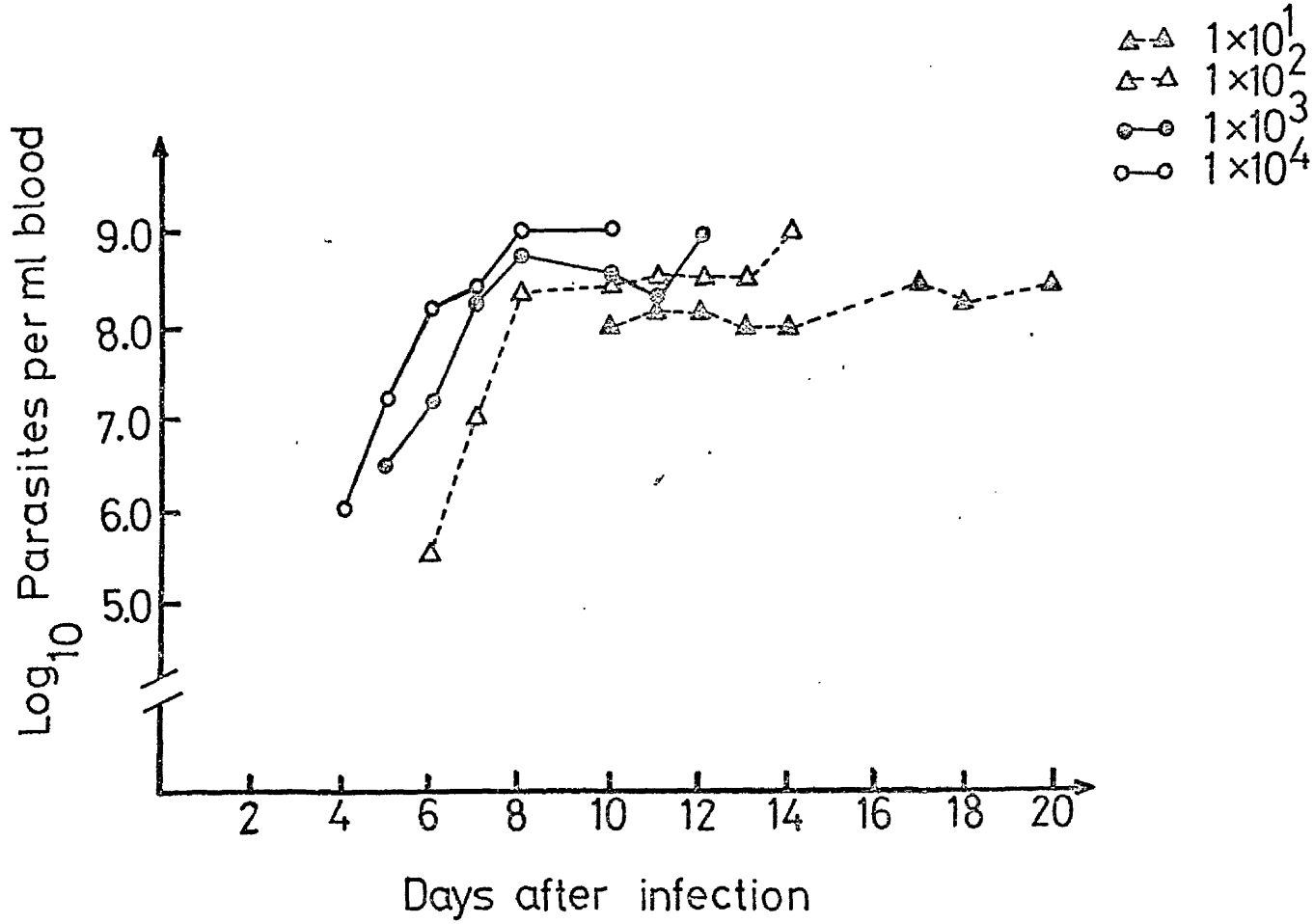


Figure 4.6 The effect of levamisole(A), FCA(B), C.parvum(C) and BCG(D) on the pattern and of T.congolense in CFLP mice

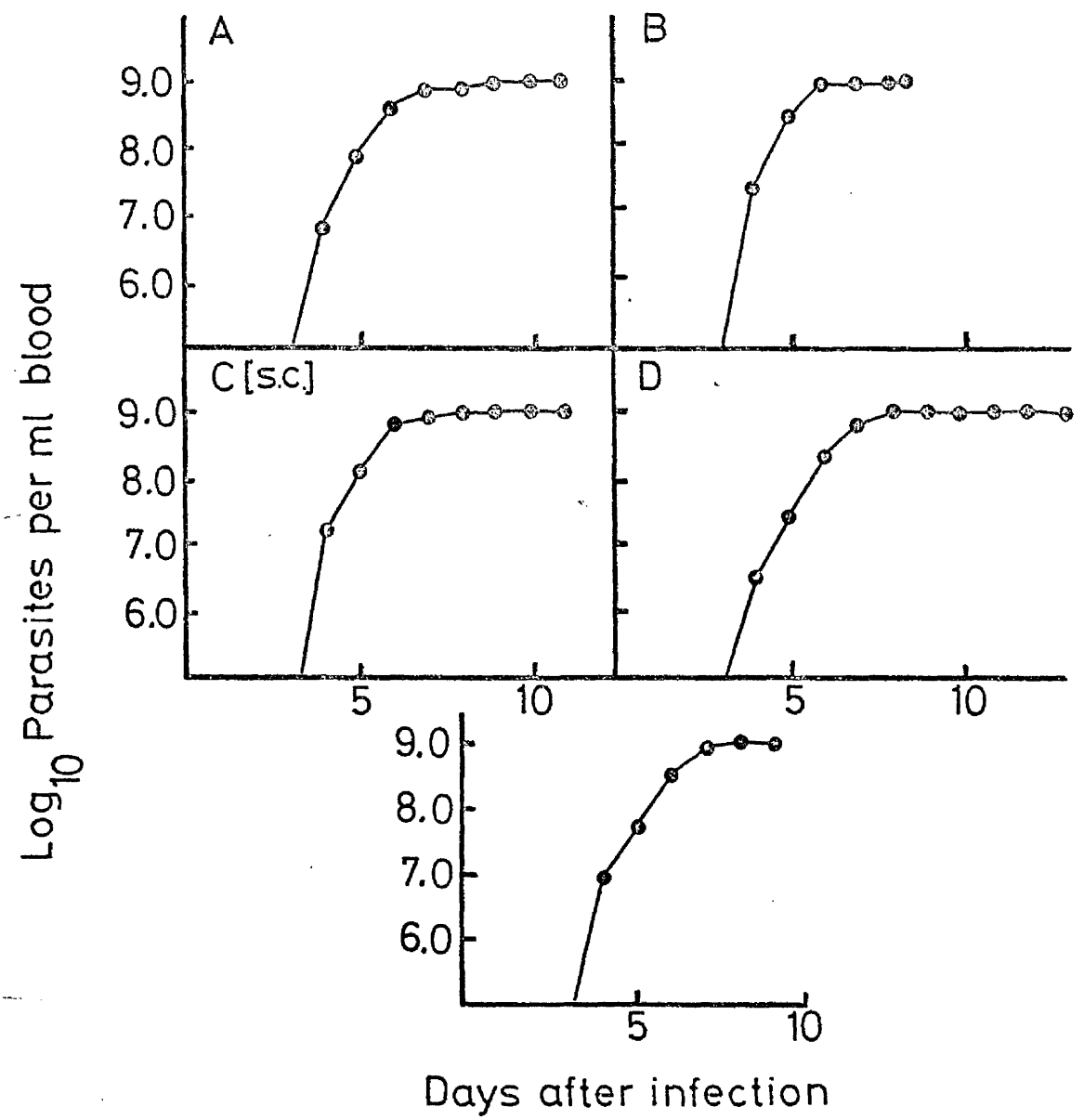
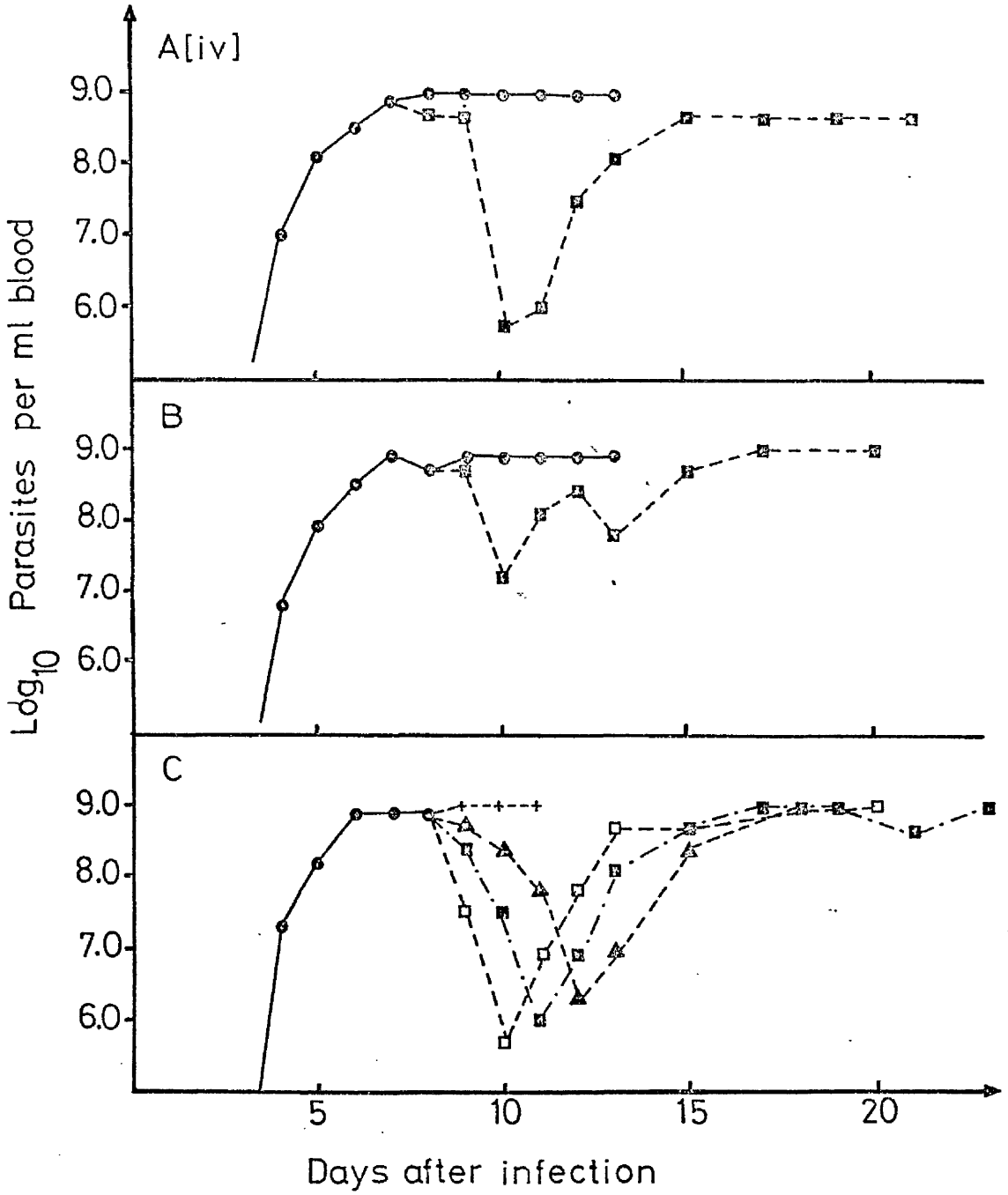


Figure 4.7 The effect of C.parvum (A), B.pertussis (B) and E.coli LPS (C) on the pattern and course of T.congolense in CFLP mice



The results presented in Fig. 4.6 show that none of the following:- levamisole, FCA, C. parvum (s.c.) and B.C.G. gave any prolongation of the infection in CFLP mice. However, C. parvum (i.v.) B. pertussis and E. coli LPS (Fig. 4.7) did show some regulating influence although the majority of animals in each group died within the normal period.

Thus C. parvum enabled one mouse out of 5 to control the first peak parasitaemia, although it eventually died by day 20, following relapse. Similarly, B. pertussis enabled one mouse out of 5 to control the first peak. However it too died by day 20 following relapse.

E. coli LPS was the most successful in producing a period of remission in that 3 out of 5 mice controlled the first parasitaemia. However, again all mice died between days 18 and 20 following relapse.

Thus hyperplasia and hypertrophy of the MPS alone does not confer any significant degree of protection in the absence of an effective antibody response.

Discussion.

The limitations associated with conventional tsetse and trypanosomiasis control, together with the unlikely possibility that an effective vaccine against the parasite will be forthcoming in the near future, has made trypanotolerance or genetic resistance an attractive alternative.

Trypanotolerance has been shown to occur in cattle (Stewart, 1951; Desowitz, 1959; Toure, 1977; Murray, Morrison, Murray, Clifford and Trail, 1979) and in sheep and goats (Toure, 1977; Griffin, 1978; Griffin and Allonby, 1979). Recently certain strains of mice have been shown to be more resistant to T. congolense infection, notably C57Bl mice (Ssenyonga/

(Ssenyonga, 1974; Jennings, Whitelaw, Holmes & Urquhart, 1978; Morrison, Roelants, Mayor-Withey and Murray, 1978). Although the basis of trypanotolerance is not known, initial studies have suggested that it is not dependent on the ability to limit physiological or biochemical derangements (Whitelaw et al, 1980).

The findings presented in this chapter suggest that the basis of genetic resistance, at least in the mouse model, is the result of a more efficient immune response, in particular the ability to maintain serum IgM levels.

From the studies of Jennings et al (1978) and the results of the initial experiment in this chapter, C57Bl mice were shown to be able to control the initial parasitaemia. In addition, closer examination of the increases in the peripheral parasitaemia in both strains suggests that the rate of growth in peripheral parasitaemia may be an important factor in determining the outcome of the infection. Thus, in C57Bl mice the rate of growth in peripheral parasitaemia slows from day 5 onwards, while CFLP mice show an almost continual growth in peripheral parasitaemia.

However, it is known that immunosuppression of mice normally able to control the peripheral parasitaemia, by sub-lethal irradiation or by cyclophosphamide therapy (Vickerman, Sless, Haston and Edwards, 1977; Whitelaw, 1979; Hudson and Terry, 1979) abolishes the ability to control the peripheral parasitaemia and such animals die with a fulminating parasitaemia. The integrity of the host's immune response would therefore appear to be important in resistance to infection.

Using/

Using ^{75}Se-labelled T. congolense to assess the immune response in CFLP mice and C57Bl mice to T. congolense infection, it was shown that CFLP mice were unable to remove radiolabelled parasites from their blood. In contrast, C57Bl mice could remove a large percentage of the injected activity by hepatic uptake. These differences were confirmed by infectivity neutralisation and trypanolysis of radiolabelled parasites. Such results indicate that the ability of C57Bl mice to control the initial parasitaemia may be dependent on the levels of effective circulating antitrypanosome antibody.

Whether trypanolysis is an important mechanism of parasite removal in vivo is uncertain. Recently Klaus, Pepys, Kitajima and Askonas (1979) have shown that mouse complement is only poorly activated by IgM, while guinea pig serum is readily activated by IgM. Thus the high trypanolytic activity of serum from infected C57Bl mice, in the presence of GPS, in vitro may not necessarily reflect a similar situation in vivo. However, it may indicate that a more effective antibody response is occurring in C57Bl mice.

When immunoglobulin levels were measured over the initial 12 days following infection, distinct differences were found in the response of C57Bl mice and CFLP mice. Thus the primary immune response in C57Bl mice, as measured in terms of IgM production, was considerably longer than in CFLP mice. In addition, the decline in IgM production seen in CFLP mice around day 7 occurred at a time when C57Bl mice were limiting the initial rise in peripheral parasitaemia.

There are several reports to suggest that IgM is important in the protective immune response in trypanosomiasis (Murray et al, 1979; Hudson and Terry, 1979) and Clarkson (1976b)has also shown that a marked increase/

increase in IgM occurs in C57Bl mice, while there is virtually no increase in highly susceptible C3H/mg. mice.

However, during trypanosome infections the immune response to heterologous antigen is depressed and on the basis of this it has been suggested that the response to trypanosome antigen may also be suppressed. Yet it is known that chronically infected animals are still able to limit relapsing parasitaemias and Hudson and Terry (1979) have suggested that this is achieved by the small residual production of IgM. While the results presented in this chapter show that both C57Bl mice and CFLP mice are immunosuppressed to SRBC presented five days after infection, they also indicate that C57Bl mice are less severely affected by the parasite induced immunosuppression. Only IgM (direct) PFC responses were measured and these results support the finding that the ability to control peripheral parasitaemia may be dependent upon the levels of circulating anti-parasite IgM. IgG production is dependent upon T cell co-operation, while IgM production is almost completely independent of such co-operation (Dresser, 1972). Furthermore, the response to SRBC is thought to be dependent upon T cell involvement (Claman & Chaperon, 1969; Katz and Benacerraf, 1972). During a trypanosome infection an abnormal T cell response may affect the response to SRBC and other T cell dependent antigens, while allowing T independent antigen responses. Mansfield and Bagasra (1978) have shown that the response of trypanosome infected mice to T independent antigens was, if anything, enhanced, although Murray et al (1974a) have shown that mice infected for 3 weeks were unable to respond to the B-cell mitogen E. coli LPS.

However, chronically infected animals can repeatedly limit the peripheral parasitaemia of relapse variants. Furthermore, the ability of chronically infected C57Bl mice to respond to an irradiated trypanosome vaccine/

vaccine, as measured by the hepatic uptake of ^{75}Se-labelled trypanosomes, confirms that an immune response to trypanosome antigen can be produced. In contrast, Whitelaw et al (1980) have shown that C57Bl mice at a similar stage of infection do not produce a detectable haemagglutin response to HRBC.

These findings suggest that an effective antitrypanosome response can be produced even when the response to HRBC and SRBC are suppressed. The nature of the antigen is therefore important and the dependancy upon T cell regulation may influence the efficiency of the immune response during trypanosome infections. This suggests that trypanosome antigen elicits a mainly IgM response that may not be under such rigorous T cell control as are SRBC responses. Indeed it has been shown that the immune response to trypanosomiasis is relatively independent of T lymphocytes (Campbell, Esser and Phillips, 1978; Clayton, Ogilvie and Askonas, 1979). In these experiments athymic mice were able to mount an effective immune response to trypanosome infection, as judged by either their response to vaccination (Campbell et al, 1978) or the similar pattern and course of infection compared to normal mice (Clayton et al, 1979).

It is difficult to explain the failure of passive immunisation using serum which had been shown to be protective using a variety of in vitro techniques (vide supra). It is possible that by injecting immune serum, which may contain antibody/antigen complexes, the B cells responsible for antitrypanosome responses may be blocked (Oberbarnscheidt and Kolsch, 1978) or that levels of IgG, when HIS was used, inhibited the production of IgM and thus reduced the effectiveness of the immune response (Finkelstein and Uhr, 1964). Indeed in vitro assessment of protective ability does not take into consideration the possibility that antigenic variation may rapidly eliminate the protective ability of the serum in vivo (Balber, Bangs, James and Proia, 1979).

The/

The susceptibility of a host to infection does not seem to be dependent on the number of infecting organisms. This may suggest that the ability to mount an effective immune response is dependent upon an intrinsic mechanism, possibly the prevention of antibody producing cells switching from IgM to IgG production, rather than the speed of the immune response.

Finally, Murray and Morrison (1979) have suggested that non-specific stimulation of the MPS can produce significant changes in the susceptibility of mice to T. brucei and T. congolense infections. In contrast, the results presented in this chapter show that in only one case (E. coli LPS) did an appreciable alteration to the parasitaemic profile occur. Indeed, the susceptibility of the mice was only reduced to the initial parasitaemia as these mice succumb to a fulminating relapse parasitaemia. The ability to increase the activity of the MPS in the absence of effective levels of antibody does not therefore reduce susceptibility. E. coli LPS may have facilitated partial control of the initial parasitaemia by activation of B cells producing anti-trypanosome antibody, as it is a recognised B cells mitogen in vitro. Indeed the failure of levamisole hydrochloride to alter the course of infection may be taken to support the concept that T cell involvement is of limited importance in the immune response to trypanosomes. Levamisole acts by restoring cell mediated immune mechanisms and is therefore of therapeutic use in parasitic infections such as leishmaniasis (Symoens, 1980).

Furthermore, in mice which have reduced the initial parasitaemia the process of antigenic variation presumably produces a new variant to repopulate the bloodstream. This relapse population of trypanosomes will initiate/

initiate a new primary immune response and the ability to maintain high serum levels of IgM will once again become the principal factor in determining the outcome of the infection. This variant population will have a similar pattern and course as the primary infection and will result in a fatal infection in susceptible mice. Thus the use of immunopotentiating agents, although successful over the initial parasitaemia, will be unlikely to assist the host in controlling subsequent variant populations.

Overall the findings presented in this chapter suggest that the ability to limit and reduce the peripheral parasitaemia is a crucially important factor in determining the susceptibility of an animal to trypanosomiasis. This ability is apparently associated with the maintenance of high levels of plasma IgM. Although immune responses to heterologous antigens are suppressed, it is questionable whether the response to trypanosomes is as seriously affected.

Therefore the genetic resistance of C57Bl mice to trypanosome infection is apparently due to an ability to limit repeatedly the peripheral parasitaemia which never reaches the absolute numbers of circulating parasites observed in susceptible CFLP mice. This is achieved by a more effective immune response, possibly dependent on the higher levels of IgM produced by these mice.

Chapter 5

IMMUNOSUPPRESSION IN BOVINE TRYPANOSOMIASIS: RESPONSE OF *T. CONGOLENSE* INFECTED CATTLE TO FOOT AND MOUTH DISEASE VACCINATION

Introduction.

The observation by MacGregor and Barr (1962) that children with malaria responded less well to tetanus toxoid than non-infected children was the first clear evidence that immunosuppression could be an important consequence of protozoal infections. Subsequently Goodwin (1970)and Goodwin et al (1972) using sheep erythrocytes showed a depressed haemagglutination response in experimental mice infected with T. brucei.

Since then it has been repeatedly confirmed that trypanosomiasis in small laboratory rodents causes a dramatic reduction in the immune responsiveness to heterologous antigens. Similarly, several workers have reported a reduction in the number of plaque forming cells present in infected mice (Murray et al, 1974a; Freeman, Hudson, Byner & Terry, 1974; Hudson et al, 1976; Corsini et al, 1977).

Whether cell mediated responses during trypanosome infection are similarly depressed remains controversial. Thus, while Murray et al (1974) using oxazolone and Jennings et al (1974) with I^{125} deoxyuridine found no significant reduction in either delayed type hypersensitivity or in the primary responsiveness of T cells to oxazolone, others (Mansfield and Wallace, 1974) using T. congolense or T. gambiense (Ackerman and Seed, 1976), have found a reduction in the delayed type hypersensitivity reaction to mycobacterium protein and oxazolone respectively. Murray et al (1974a)however did find a significant depression to oxazolone during the terminal stages of the infection in mice.

The potential significance of immunosuppression was demonstrated by Urquhart, Murray, Murray, Jennings and Bate (1973) in rats doubly infected with T. brucei and an intestinal nematode Nippostrongylus brasiliensis. In such animals the trypanosome infection significantly delayed/

delayed and in some cases prevented the normal immune expulsion of the nematode infection from the small intestine. Furthermore, Murray et al (1974b)have also shown that if N. brasiliensis infected rats were allowed to undergo self-cure and subsequently infected with trypanosomes, this prevented expulsion of a challenge N. brasiliensis infection. Thus trypanosomiasis may suppress both primary immune and secondary immune responses.

The importance of trypanosome-induced immunosuppression in natural bovine and ovine infections has received little attention until recently compared to the considerable amount of work on this phenomena in laboratory rodents. There are, however, many anecdotal reports on the increased susceptibility of trypanosome infected domestic animals to secondary bacterial infections, together with a few experimental results (Parkin and Hornby, 1930; Hull, 1971).

The first attempt to evaluate the significance of trypanosome-induced immunosuppression in cattle under experimental conditions was reported by Holmes, Mammo, Thomson, Knight, Lucken, Murray, Murray, Jennings and Urquhart (1974). Using Ethiopian Zebu cattle from a tsetse-free area these workers were able to demonstrate a reduced secondary response to vaccination with a clostridial vaccine in trypanosome infected animals. In these experiments two groups of cattle were first vaccinated with a polyvalent clostridial vaccine which produced a similar primary immune response in both groups. One group was subsequently infected with T. congolense and three weeks later both groups were revaccinated. It was found that the antibody titres in the trypanosome infected cattle were considerably lower than those in control animals and, although the differences were less dramatic than those observed in laboratory animals, they suggested that trypanosome induced immunosuppression could possibly interfere with/

with the vaccination campaigns and predispose cattle to other infections.

Mackenzie, Boyt, Emslie and Swanepoel (1975) undertook a similar experiment in sheep following their observation that sheep infected with T. congolense were often killed by secondary bacterial pneumonia. In their experiment, groups of Blackhead Persian sheep were infected with T. congolense and seven days later when there was a patent parasitaemia the animals, together with controls, were vaccinated with Vibrio foetus antigen. On day 21 both groups received a second vaccination of V. foetus. The results showed that both primary and secondary humoral responses to V. foetus antigen were significantly depressed in infected sheep.

The most recent series of experiments are those described by Scott, Pegram, Holmes, Pay, Knight, Jennings and Urquhart (1977). In contrast to the previous two reports in which the infections were experimentally initiated, this series of experiments describe the effect of natural infection on the antibody responses to polyvalent Foot and Mouth Disease (FMD) vaccine and a polyvalent clostridial vaccine in oxen maintained in a trypanosome endemic area. The infected animals were continuously exposed to tsetse challenge and were parasitaemic at the time of vaccination, whilst the control cattle were maintained in the same area but were protected from infection by fortnightly Berenil therapy. A polyvalent FMD vaccine was administered in two doses 21 days apart and in some groups a polyvalent clostridial vaccine was also inoculated at the same time. Subsequent serum antibody tests showed that there was a marked reduction in the immune response to FMD vaccine in trypanosome infected animals compared with non-infected controls. However the levels in both groups were still considered to be adequate for successful protection to needle challenge with FMD virus according to the FMD titres previously reported to confer 95 per cent protection

(Pay/

(Pay and Parker, 1977).

The immune response to FMD vaccine was also delayed in trypanosome infected cattle which did not develop protective FMD titres until day 31 in contrast to control cattle which achieved protective titres by day 21.

Thus there was considerable evidence to suggest that immunosuppression was associated with both experimental and natural trypanosome infections of cattle and could be a significant factor in the efficacy of vaccination in trypanosome endemic areas.

Whilst such studies in Africa were valuable, they suffered from several constraints. First, since FMD is endemic in most of Africa including Ethiopia, there was a possibility that the cattle had been previously exposed to FMD. Secondary, due to local difficulties it was not possible to measure the duration of immunity following vaccination of infected cattle. Thirdly, the level of protection in vaccinated animals could not be assessed by live virus challenge because of veterinary restrictions. Such contraints would not apply to experimental studies on cattle kept at a FMD research unit in Britain.

This chapter describes an initial series of collaborative experiments with the Animal Virus Research Institute (AVRI), Pirbright, Surrey.

The experiments were designed to measure the development and duration of the humoral responses to FMD vaccination under carefully controlled conditions. In addition, they offered a unique opportunity to assess the level of protection in vaccinated animals by direct challenge with live FMD virus.

FMD type O and C vaccines were used widely throughout Africa and were therefore the first choice in these experiments.

Materials/

Materials and Methods.

Parasite:

Trypanosoma congolense GVR 1 was used in all experiments. In addition T. congolense GVR 17 was used in the first experiment. As stated in the general material and methods, the history of T. congolense GVR 1 is unknown. T. congolense GVR 17 was isolated from an infected bovine near Mazabuka, Zambia, and imported to Glasgow in mouse blood late in 1978. Since that time it has been stored as a stabilate in liquid nitrogen and used regularly to infect mice.

Experimental Animals:

The experimental animals used in these experiments were 9 month old Friesian steers and Mature Devon Longhorn steers of approximately three years of age. All the animals had water available continually and were fed on a diet of Lucerne cobs and hay (British Co-op. Driers Ltd., Lincoln). They were housed in an isolation block at the AVRI, Pirbright, Surrey, on concrete floored loose boxes with no bedding.

Trypanosome Infection:

Each animal was infected with 5×10^5 trypanosomes prepared from stabilate blood in PBGS administered by subcutaneous injection behind the shoulder.

Foot and Mouth Disease (FMD) vaccination:

Two different commercial monovalent FMD vaccines were used in these experiments (Wellcome Foundation Ltd., Surrey).

In young 9 month Friesians type C vaccine (C_1 Noville batch 326, U.K. Strategic Reserve) and adult Devon Longhorn cattle type O vaccine (O_1 BFS 1860, batch 344, U.K. Strategic Reserve) were used.

Chemotherapy:

When required, steers were given 3.5 mg. active principle diminazene/

diminazene aceturate (Berenil, Fabwerke, Hoechst, Germany) per kilogram body weight by intramuscular injection.

Estimatation of Parasitaemia:

Blood parasitaemia was measured by recording the number of parasites in 20 fields. When a parasitaemia could not be detected by this method, the buffy coat was examined for the presence of parasites after the estimation of packed cell volume (PCV) using the microhaematocrit (Hawksley & Sons Ltd., London).

FMD Assay:

Serum antibody titres of cattle to FMD virus vaccination were measured by the method of Golding, Hedger, Talbot and Watson (1976).

Iodination of bovine gamma globulin:

Bovine gamma globulin (Fraction II, Armour Pharmaceutical Company Ltd., Eastbourne) was iodinated with 1mCi of I^{125} by an iodine monochloride method (Macfarlane, 1958). Each animal was intravenously injected with a known amount of I^{125} gamma globulin into the right jugular vein. Samples were collected from the left jugular regularly over a 10 day period.

Live FMD virus challenge and assessment of lesions:

10^5 virus particles, prepared from stock glycerinated filtrate of bovine tongue epithelium stored at $-20^{\circ}C.$, were injected intradermalingually and the animals monitored for the development of secondary lesions. Whether an animal was protected from the virus was based on its ability to limit lesions to the tongue and lips. Non-protected animals showed one or more foot lesions within 48 hours of challenge.

Experiment I: Pattern and course of T. congolense infection in calves.

Animals	Infection	Berenil Treatment	^{125}I IgG	Killed
9 mth. Friesians	Day 0	Day 45	Day 211	Day 235
1	T. congolense GVR 1	+	+	+
2		-	+	+
3	T. congolense GVR 17	-	+	+
4		+	+	+

Experiment II: Antibody response to FMD vaccination in adult cattle infected with T. congolense.

Animals	Infection	Primary Vaccination	Secondary Vaccination
Adult Devon Longhorn	Day 0	Day 21	Day 35
5 6 7 8	T. congolense GVR 1	Vaccinated FMD Type 0 vaccine	Vaccinated FMD type 0 vaccine
9 10 11 12	-		

Experiment III: Antibody response and ability to resist live virus challenge after FMD vaccination of calves infected with T. congolense.

Animals	Groups	T. congolense GVR 1 Infection	1° Vacc.	Berenil	Live Challenge	2° Vacc.	Killed
		Day 0	Day 21		Day 42	Day 83	Day
9 months Friesian steers in all groups	A	+	+	-	-	+	104
	B	+	+	-	+		45
	C	+	+	+	+		45
	D	-	+	-	+		45
	E	-	+	-	-	+	104
	F	-	-	-	+		45

Experimental Protocol.

Results.

Trypanosomiasis in young cattle - Exp. Ia:

This experiment was designed to investigate the ability of two strains of T. congolense to infect young cattle and the response of these parasites to chemotherapy. T. congolense 1 is a stabilate of extended experimental and syringe passage and its infectivity to a bovine host was uncertain. In contrast, T. congolense 17 was a recent bovine isolate but its suceptibility to chemotherapy was unknown. As future experimentation was dependent upon the ability of Berenil to prevent relapse, it was important to establish the susceptibility of both strains to this drug.

The PCV and parasitaemic profiles of these infections are presented in Figs. 5.1 and 5.2. Figure 5.1 illustrates the pattern of the T. congolense 1 infection in steers 1 and 2. Both steers became parasitaemic between days 7 and 10. After peak parasitaemia there was a short period of remission followed by a series of relapsing parasitaemias. The PCV, initially between 0.3 and 0.35, fell over the early period of infection until by the third week it was between 0.15 and 0.2.

During the initial three weeks of infection each relapsing parasitaemia lasted between 3 and 4 days from first appearance of the parasites in the blood to their absence from the circulation. However, after this initial period the duration of each relapse population became irregular and the numbers of circulating parasites was lower and less consistent. This change in parasitaemic profile was mirrored by a stabilisation of the anaemia. The bodily condition of the animals which had deteriorated over the initial period also began to recover during this period of the infection.

When steer 1 received Berenil on day 45 of the infection, the parasites/

Figure 5.1 The parasitæmic (▲—▲) and PCV (o—o) profiles of T.congolense GVR1 infected cattle

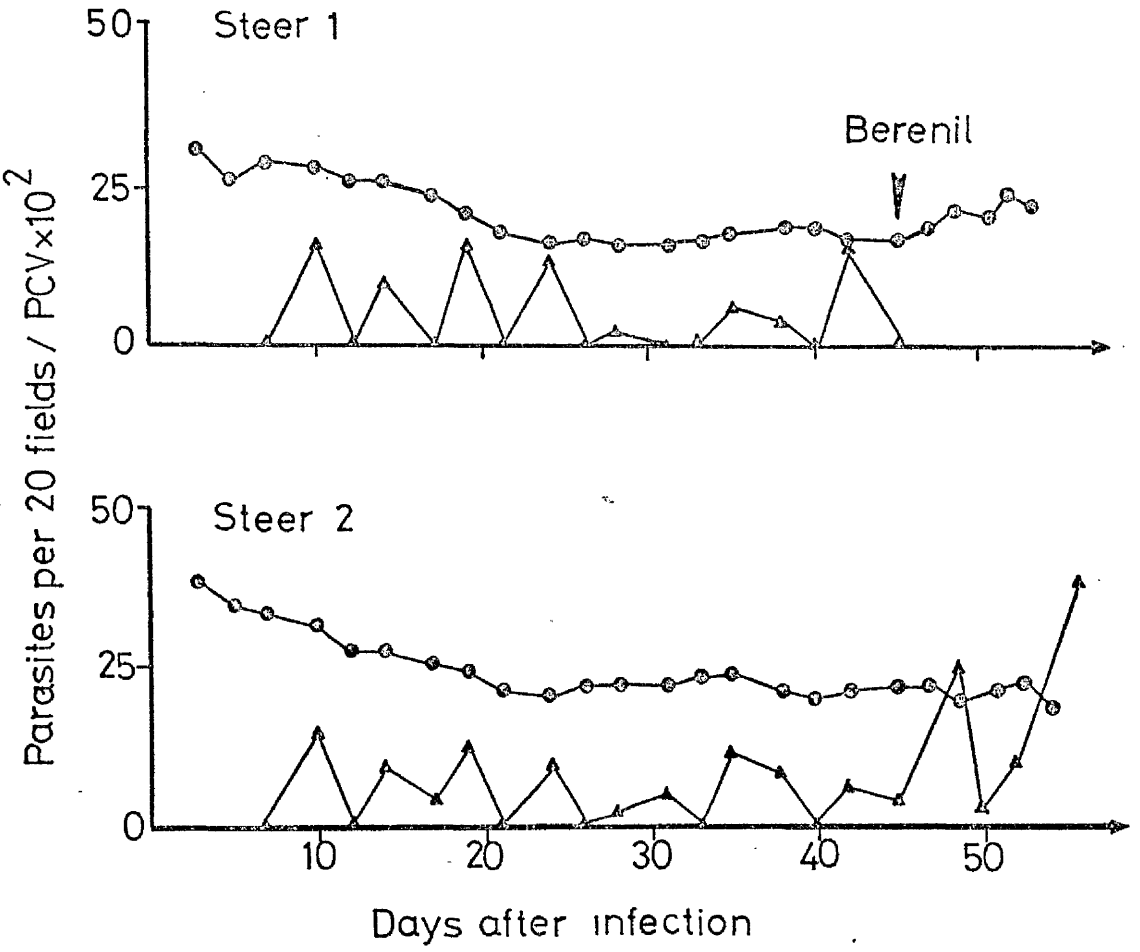
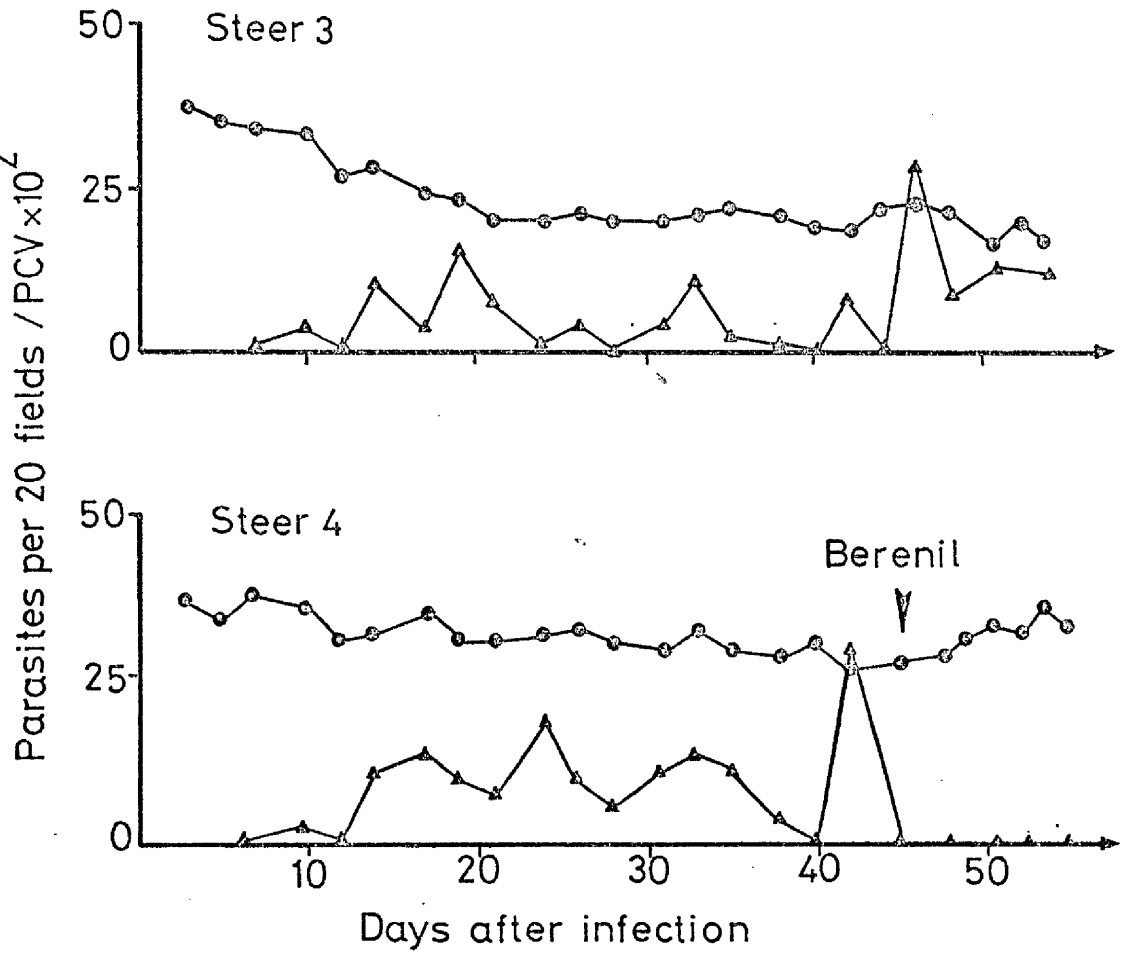


Figure 5.2 The parasitaemic (▵—▵) and PCV (⊙—⊙) profiles of T.congolense GVR 17 infected cattle



parasites rapidly disappeared from the circulation and this animal remained free from peripheral parasitaemia for the remainder of the experiment. The removal of parasites by chemotherapy was accompanied by a rapid recovery of the haematocrit until by 6 days following chemotherapy it was 0.25.

Figure 5.2 illustrates the PCV and parasitaemic profiles of steers 3 and 4 infected with T. congolense 17. The parasitaemic profiles were similar in both steers with a characteristic mild initial parasitaemia between days 8 and 14. Each parasitaemic wave was longer in duration than that of T. congolense 1 lasting approximately 8 days in the peripheral circulation. Consequently the number of different relapse populations during the course of these experiments was fewer in T. congolense 17 infections, i.e. 4 as opposed to 7 in T. congolense 1 infection up to day 45.

The PCV profiles for these two steers were, however, different. Steer 3 had an initial PCV of 0.37 which by day 21 had fallen to 0.20. The PCV then stablised at this level. In contrast, the infection of steer 4 produced no significant fall in the PCV which remained around 0.30. After Berenil treatment of steer 4 the peripheral circulation remained free of parasites for 24 days after which time they reappeared. The haematocrit after chemotherapy showed little change.

On day 211 of the initial infection steers 1, 2, 3 and 4 were used in a bovine gamma globulin turnover experiment.

Trypanosomiasis in adult cattle - Expt. IIa:

In view of the success of establishing a chronic infection in young cattle with T. congolense 1 and its susceptibility to Berenil therapy, it was decided to use T. congolense 1 in preference to T. congolense 17 for subsequent cattle experiments.

A/

Figure 5.3 The parasitaemic (△—△) and PCV (o—o) profiles of T.congolense infected cattle

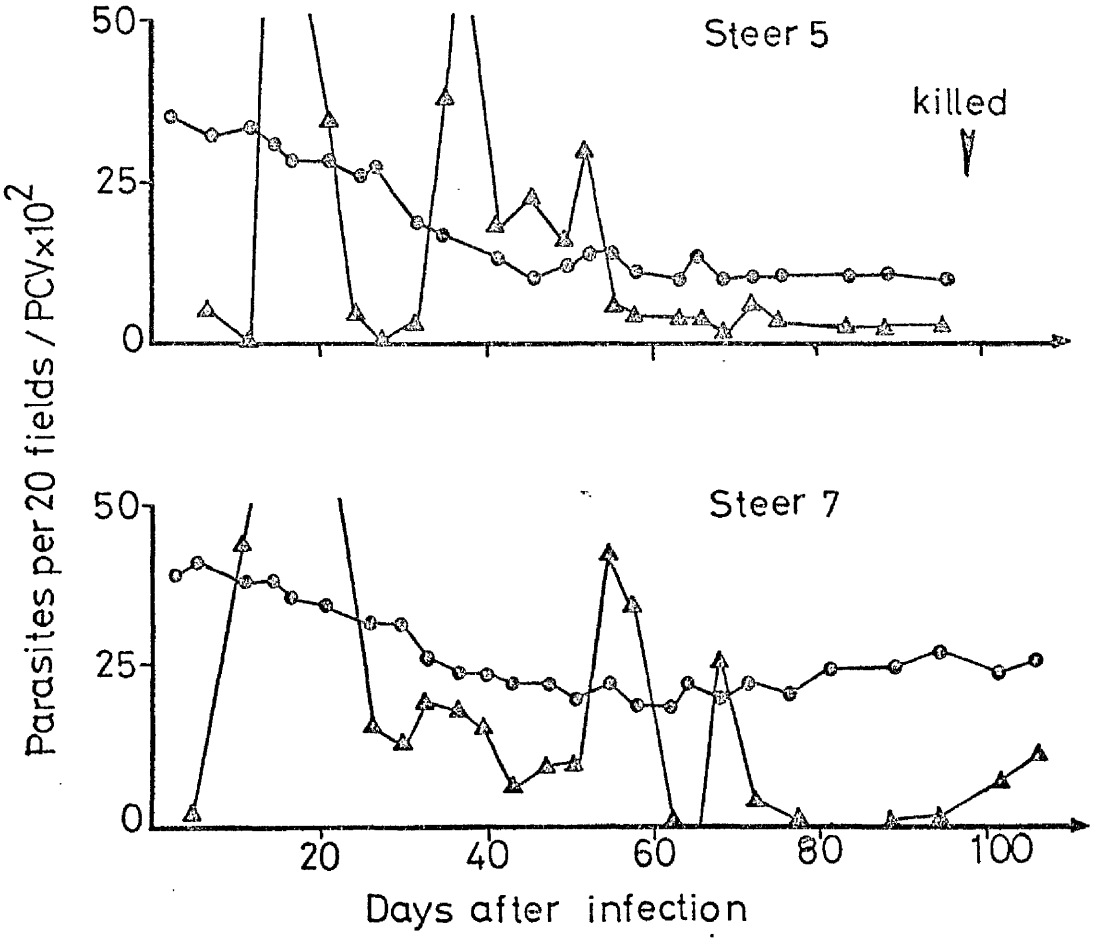
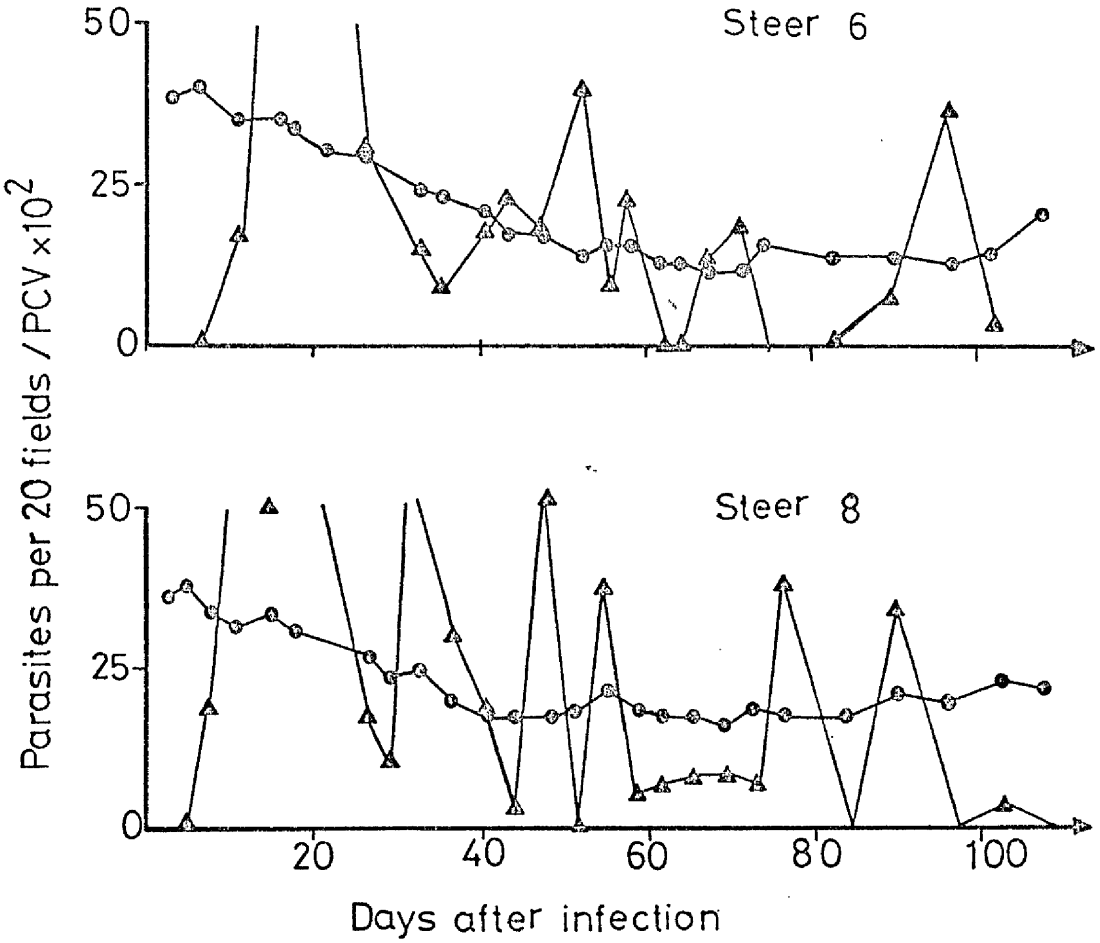


Figure 5.4 The parasitæmic (Δ—Δ) and PCV (o—o) profiles of T.congolense infected cattle



A group of 4 adult Devon Longhorn steers were infected with T.congolense 1 and their parasitaemic and PCV profiles studied. The results are presented in Figs. 5.3 and 5.4.

All steers became parasitaemic by day 8 and developed a characteristic relapsing parasitaemia. Two animals, 5 and 7, developed a chronic infection starting between days 58 and 62 after infection which produced a continually low peripheral parasitaemia while steers 6 and 8 developed an infection with more noticeable fluctation in peripheral parasitaemia.

Several interesting features in the PCV were associated with these parasitaemia profiles. In only one animal, steer 7, was the development of the anaemia restricted and a stabilisation of the PCV occurred. The PCV, initially between 0.38 and 0.4, fell by day 48 to approximately 0.2, then stabilised and subsequently began to recover from day 76 onwards. This stabilisation was associated with a low peripheral parasitaemia. The PCV finally remained around 0.25. In all other animals the PCV fell continually over the period of infection. The fall in PCV was most dramatic in steer 5 reaching values of 0.12 and the bodily condition of this animal deteriorated so markedly that it was killed on day 97 after infection. The peripheral parasitaemias were considerably higher in these cattle than that observed in young animals. In addition, the degree of anaemia was apparently directly related to the level of this initial parasitaemia.

The general bodily condition of all the animals was poor and was associated with clinical features characteristic of natural infections such as a staring rough coat and lachrymation.

The adult cattle were clearly more severely affected by trypanosomiasis then were the young steers, and this enabled a comparison to be made between animals with different levels of clinical/

clinical disease and their ability to maintain an immune response to FMD vaccine.

The immune response to FMD vaccination and its duration in young cattle infected with T. congolense GVR 1 - Expt. III:

This experiment was designed to investigate the primary and secondary immune response to FMD vaccination and the duration of this immunity in infected and non-infected animals.

(a) Primary immune response.

A group of five 9-month old Friesian steers were infected with T. congolense 1 (A) and 21 days later vaccinated with FMD type C vaccine together with five control animals (E). Serum samples were collected at regular intervals and assayed for antibody against FMD virus. The results are presented in Table 5.1. The small numbers of cattle per group make statistical evaluation difficult but the infected group of animals did have a mean FMD titre consistently lower than that found in non-infected vaccinated cattle. The maximum response to vaccination also developed later in infected animals than in non-infected animals. Thus a maximum response of $\log_{10}$ 1.06 developed by day 21 in infected cattle whilst a maximum response of 1.56 developed by day 15 in non-infected animals. The duration of the primary response to vaccination was not altered by trypanosome infection. However by day 83 the mean titre of 1.2 in infected animals was still lower than the mean titre of 1.5 found in non-infected animals. This difference may be sufficient to compromise the infected animal to live challenge.

(b) Secondary immune response.

These two groups of cattle were revaccinated 83 days after the primary vaccination. Again the mean log titre in infected animals was consistently lower than those in non-infected animals. In both groups, however, the level of antibody in their serum would probably be sufficient to confer 95% protection to live virus challenge (Pay and Parker, 1977).

(c)/

TABLE 5.1 The antibody response of T. congolense infected calves following vaccination with FMD type C vaccine.

Group	1st Vaccination(days)		0	8	15	21	50	83	90	97	104
	2nd Vaccination(days)							0	7	14	21
A	Trypanosome infected (21 days)	0	0.78*	0.9	1.34	1.34	1.5	1.65	2.41	2.85	2.41
		1	0.78	0.78	0.78	0.9	0.78	0.9	1.5	2.5	2.25
		2	1.34	1.5	1.5	1.5	1.20	1.5	1.81	3.01	2.71
		3	0.78	0.78	0.78	0.78	0.78	0.9	1.81	2.5	2.41
		4	0.78	0.78	0.78	0.78	1.04	1.04	2.11	2.85	2.41
			0.89	0.95	1.04	1.06	1.06	1.2	1.93	2.74	2.44
E	Control	20	0.78	1.04	1.34	1.34	0.78	1.34	2.41	2.85	3.01
		21	0.78	0.78	1.04	0.78	0.78	1.34	2.85	3.01	3.01
		22	0.78	1.34	1.34	1.2	1.34	1.65	2.5	3.01	3.01
		23	0.78	1.5	2.11	1.95	1.81	1.81	2.71	3.15	2.71
		24	0.78	1.5	1.95	1.65	1.34	1.34	2.5	3.15	2.71
			0.78	1.23	1.56	1.39	1.27	1.5	2.59	3.03	2.89

* Log_{10} titres.

(c) Response to live challenge.

A direct assessment of the protection conferred by vaccination can be obtained by measuring the ability of cattle to withstand a challenge with live virus. The nature of the quarantine accommodation at Pirbright enabled such an evaluation to be carried out.

Two groups of five 9-month Friesian steers were infected with T. congolense 1 (B and C) and after 21 days these two groups, together with 5 control steers (D), were vaccinated with FMD type C vaccine. One of the infected groups of cattle was treated with Berenil at the time of vaccination (C). Serum samples were collected regularly and assayed for protective antibody against FMD virus. All the animals in each group were then challenged with 10^5 live virus particles 21 days after the primary vaccination, together with 5 non-infected non-vaccinated steers (F), and the appearance of lesions monitored. The results for antibody titre and reaction to live challenge are presented in Table 5.2.

The antibody response was again consistently lower in both infected groups (B, C) when compared to non-infected cattle (Group D). Furthermore, Berenil therapy did not affect the level of detectable antibody in infected cattle.

The reaction to live challenge suggested that the protective immune response in infected cattle had also been reduced. Thus three out of five steers from the infected group (B) were not protected while two out of five were not protected in the group of infected animals which received Berenil at the time of vaccination (C). In contrast, four out of five non-infected control vaccinated animals (Group D) were protected while all of the non-infected, non-vaccinated animals developed severe lesions (Group F).

Immune/

TABLE 5.2 The antibody response and result of live virus challenge in T. congolense infected calves.

Group	1st Vaccination(days)		0	8	15	21	Response to live challenge on day 21			
							Tongue	Lips	No. of feet	Protected?
B	Trypanosome infected(21 days)	5	1.04*	0.78	0.78	1.2	4+	+	0	+/-
		6	0.78	0.78	0.78	0.78	4+	-	4	-
		7	0.78	1.2	1.81	1.65	3+	-	0	+
		8	0.78	0.9	1.34	1.34	4+	-	2	-
		9	1.2	1.2	0.9	1.5	4+	+	1	-
			0.92	0.97	1.12	1.29				
C	Trypanosome infected(21 days) Berenil at vaccination	10	0.78	0.78	0.78	0.78	4+	+	4	-
		11	0.78	0.78	0.78	0.78	4+	+	4	-
		12	0.78	1.65	1.5	1.5	3+	-	0	+
		13	0.78	1.2	1.34	1.34	4+	-	0	+
		14	0.78	1.04	1.2	0.9	4+	-	0	+
			0.78	1.09	1.12	1.06				
D	Control	15	0.78	0.90	1.95	1.81	3+	-	0	+
		16	0.9	0.78	1.5	1.2	3+	-	0	+
		17	0.78	1.81	1.65	1.81	3+	-	0	+
		18	0.78	1.2	1.34	1.2	4+	-	0	+
		19	0.78	0.78	1.2	1.2	4+	-	3	-
			0.81	1.09	1.53	1.44				

* Log_{10} titres.

Immune response to FMD vaccination in adult cattle infected with T. congolense GVR 1 - Expt. IIb:

In contrast to the relatively mild infection produced in young cattle by T. congolense 1 which was apparently well tolerated, the disease produced in adult cattle, at least in terms of anaemia, peripheral parasitaemia and bodily condition, was more severe. This experiment was therefore undertaken to investigate the effect of this severe trypanosome infection on the primary and secondary immune response to FMD type O vaccination in adult cattle.

A group of four adult Devon Longhorn steers were infected with T. congolense 1. This group, together with four control animals, were vaccinated with FMD type O vaccine 21 days after the initial infection. Serum samples were then collected at 0, 7, 14, 21 and 28 days. The steers were revaccinated 35 days after the primary vaccination and serum samples collected at regular intervals for a further 42 days. These samples were then assayed for protective antibody and the results are presented in Table 5.3.

The primary response in infected animals was significantly lower than that of non-infected animals, although it was probably sufficient to confer 95% protection to needle challenge (Pay and Parker, 1977).

The secondary response to vaccination was again lower in infected animals than in non-infected cattle.

However, the apparent severity of the clinical signs in adult cattle was not reflected in their immune response to FMD vaccination. Thus, while the degree of anaemia was severe and bodily condition poor, the antibody responses in general were similar to those observed in infected calves.

Therefore, in both young and adult cattle with trypanosomiasis there was a general trend of reduced responsiveness to vaccination resulting in a lower maximum level of serum antibody.

TABLE 5.3. The antibody response of T. congolense infected adult cattle following vaccination with FMD type O vaccine.

Group	1st Vacc.	0	7	14	21	28	35	42	49	56	63	70	77
	2nd Vacc.						0	7	14	21	28	35	42
Tryp. infected A	5	1.51*	2.41	2.55	2.71	2.41	2.55	2.25	2.25	2.25	2.11	1.95	-
	6	0.90	2.11	1.81	2.25	1.95	1.95	2.25	2.25	2.11	1.65	1.65	1.65
	7	1.04	2.25	2.55	2.41	2.11	1.95	2.55	2.71	2.41	2.25	2.25	1.95
	8	0.78	1.34	1.65	1.65	1.65	1.51	2.25	2.11	2.11	1.95	1.81	1.65
		1.06	2.03	2.14	2.26	2.03	1.99	2.32	2.33	2.22	1.99	1.92	1.75
Control B	9	1.20	3.01	3.01	2.71	2.85	2.55	2.55	2.55	-	2.41	2.25	2.25
	10	1.04	2.55	2.71	2.55	2.55	2.55	2.41	2.55	2.25	1.95	2.25	2.25
	11	1.51	2.55	2.85	2.55	2.55	2.55	2.25	2.41	2.11	2.11	1.95	2.11
	12	1.20	2.41	2.85	2.85	2.85	2.85	2.55	2.55	2.25	2.41	2.25	2.55
		1.24	2.63	2.86	2.66	2.7	2.62	2.44	2.52	2.2	2.22	2.18	2.29

* Log_{10} titres.

Gamma globulin turnover studies in young cattle infected with GVR 1 and GVR 17 - Expt. Ib:

Immunoprophylaxis is essentially dependent upon the maintenance of protective levels of antibody in the serum and such levels may be affected by several factors such as the turnover rate of immunoglobulins, the number of exposures to antigenic stimulation, the level of challenge, generation of memory cells and the speed of the immune response. It has already been demonstrated that trypanosome infection reduces the maximum titre in response to vaccination. In addition, it has been suggested (Smithers and Terry, 1959; Jennings, Murray, Murray and Urquhart, 1973) that gamma globulin catabolism is increased during trypanosome infections and is a possible mechanism by which trypanosomiasis may further reduce the effectiveness and duration of the immune response.

The steers used in the original infection (Expt. Ia) experiments were therefore used to determine the gamma globulin turnover rate in infected cattle.

In this experiment ^{125}I-gamma globulin was injected into the jugular vein of infected cattle 211 days after infection. Serum samples were then collected at 10 mins., 20 mins., and 20 hrs. after injection and thereafter at frequent intervals over the following 19 days.

The half life of the gamma globulin for each animal was calculated by regression analysis of the plasma radioactivity plotted as a percentage of the 10 min. sample on semi-logarithmic graph paper between days 5 and 19 post-injection. The results which are presented in Table 5.4 show that the cattle has IgG half-lives ranging between 12 and 18 days. The infected cattle which had never received Berenil therapy had globulin half lives of 12.5 days, whilst steer 4 infected with T. congolense 17, which relapsed shortly after Berenil therapy, had a half life close to the infected steers of 13.9 days. In contrast,/

TABLE 5.4. The half-life of gamma globulin in *T. congolense* infected calves.

Animal	*T. congolense* infection	Berenil	Gamma globulin $\frac{1}{2}$ life (days)	Parasitaemia on day of injection.
1	GVR 1	+	17.9	-
2	GVR 1	-	12.5	+
3	GVR 17	-	12.5	+
4	GVR 17	+	13.9	+

contrast, steer 1 infected with T. congolense 1 and cured with Berenil had a half life of nearly 18 days. Thus there was some evidence to suggest that IgG catabolism is increased in animals displaying a patent parasitaemia.

Discussion.

Many parts of the developing world, especially Africa, suffer large economic losses as a direct consequence of trypanosomiasis. This chapter now demonstrates that trypanosomiasis in cattle can also reduce the effective immune response to vaccination as measured by challenge with live virus particles and confirms and extends previous findings (Holmes et al, 1974; Mackenzie et al, 1975; Scott et al, 1977; Whitelaw, Scott, Reid, Holmes, Jennings & Urquhart, 1979; Rurangirwa, Tabel, Losos, Masiga and Mulambu, 1978; Rurangirwa, Tabel, Losos & Tizard, 1980) that parasite induced immunosuppression may reduce the protective immune response of vaccination to a variety of commonly used viral and bacterial vaccines.

The initial experiment showed that T. congolense 1 was capable of infecting cattle even after extended laboratory passage. In addition, T. congolense 1 was more susceptible to Berenil therapy than was T. congolense 17. The failure of Berenil to prevent relapses in the recently isolated T. congolense 17 infection may reflect the widespread use of Berenil as a curative drug in Zambia, together with the difficulty in providing controlled veterinary supervision in recent years. These factors may have been ideal for the creation of drug resistance. T. congolense 1 was therefore chosen to infect all cattle in the subsequent experiments.

The difference in the pattern of disease between young cattle and adult cattle confirms earlier reports (Fiennes, 1970) that calves were better able to tolerate an infection than adult animals. However, these/

these differences may also have been due to differences in breed susceptibility to trypanosomiasis.

The significance of the serological response to vaccination of infected cattle remains uncertain. In all groups of cattle the primary and secondary responses were lower in infected than non-infected cattle. Nevertheless, according to Pay and Parker (1977), the levels of antibody titres achieved in infected animals after second vaccination would probably still be sufficient to confer 95% protection to challenge. In addition, the duration of the immune response, at least in terms of maintaining the level of serum antibody, did not appear to be reduced in cattle infected with trypanosomiasis.

However, in addition to the reduced production of antibody in infected animals, the response to vaccination may also be diminished by hypercatabolism of gamma globulins in such animals. Previous work (Smithers and Terry, 1959; Jennings et al, 1973) suggested that the half-life of gamma globulin in trypanosome infected animals was reduced and, more recently, Nielsen, Sheppard, Holmes and Tizard (1978) have shown a dramatic reduction in the half-life of gamma globulin in T. congolense infected cattle. It is, however, difficult to draw conclusions from the results of the ^{125}I-IgG experiment presented in this chapter due to the small numbers of animals involved and the absence of non-infected control cattle. Nielsen et al (1978) found a reduction in the half-life of IgG from approx. 20 days in normal cattle to approximately 2 days in trypanosome-infected cattle. In contrast, the results presented here show no such dramatic reduction, although they are below the normal expected values and there is a difference between animals which had received Berenil therapy and those which did not. The animals in which the gamma globulin turnover studies were performed had been infected for some

211 days and were obviously past the acute stage of the infection. Indeed, these animals were in good bodily condition, had low peripheral parasitaemias and their haematocrit was showing signs of recovery. In contrast, Nielsen et al (1978) measured serum protein half-lives over the initial period of infection when the parasitaemia was often high.

It had previously been reported from studies in Ethiopian Zebu cattle that the immunosuppressive effects of trypanosomiasis on the immune response to vaccination could be significantly reduced if Berenil was administered at the time of vaccination (Whitelaw et al, 1979). However in the present experiment no such differences could be detected between treated and non-treated infected cattle. The reasons for this discrepancy are unclear but may be due to the higher parasitaemias observed in the Ethiopian animals used in the experiments reported by Whitelaw et al (1979).

It was clear both from the results presented in this chapter and from others (Holmes et al, 1974; Mackenzie et al, 1975; Scott et al, 1977; Whitelaw et al, 1979; Rurangirwa et al, 1980a) that the level of antibody response to vaccination is consistently reduced. However whether this reduction was sufficient to affect the protective response against infection was previously unknown.

The response of trypanosome infected cattle to live FMD virus challenge 21 days after primary vaccination and 42 days after infection with trypanosomes showed that such animals were more susceptible to viral infection than non-infected vaccinated cattle. It was also found, as Whitelaw et al (1979) had suggested from monitoring the humoral response, that trypanosome infected cattle treated with Berenil at the time of vaccination were less susceptible to viral infection than non-treated/

non-treated trypanosome infected animals.

However, several authors (Scott et al, 1977; Sollod & Frank, 1979) have concluded that while a depression of the immune response occurs, it is unlikely to interfere with the actual protective levels of antibody.

In contrast, the results presented in this chapter indicate that trypanosomiasis may reduce the protective primary immune response, when assessed on the basis of resistance to live virus challenge.

This divergence of opinion as to the importance of parasite induced immunosuppression probably has its basis in the type of antigen used for its assessment and the limited number of animals involved in individual cattle experiments.

The profound degree of immunosuppression associated with experimental rodent trypanosomiasis clearly does not occur in cattle trypanosomiasis, probably largely because of the differences in peripheral parasitaemia. Furthermore, the degree of parasite induced immunosuppression may be partly dependent upon the level of cellular co-operation required to mount an effective immune response against particular antigens (see General Introduction). For example, Rurangirwa et al (1980) have shown that cattle infected with T. congolense or T. vivax produce normal immune responses to rinderpest vaccine, possibly because the rinderpest virus can by-pass the T cell co-operative stage. In contrast, T. congolense infected cattle show a reduced primary response to Leptospira biflexa vaccination (Rurangirwa et al, 1980a).

Finally, the animals used in these experiments were well maintained and under strict veterinary supervision and this may not be the situation under field conditions. In such cases mal-nourishment, prolonged foraging for food, anaemia and the continual exposure to disease/

disease may further diminish the efficiency of the immune and erythropoietic responses in trypanosome infected cattle.

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