CHEMOTHERAPY AND IMMUNITY

IN

MURINE AFRICAN TRYPANOSOMIASIS

by

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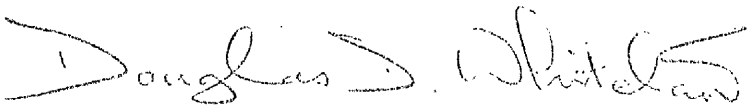
DECLARATION

The contents of this thesis are the work of the author. The thesis has not been previously submitted for the award of a degree to any university.



SUMMARY

The experiments described in this thesis investigated aspects of immunity and chemotherapy in mice infected with T. brucei and T. congolense.

It was found that mice with infections of T. brucei of less than 7 days duration were completely cured if treated at this stage with trypanocidal drugs. However, if treatment was delayed beyond 14 days, a proportion of the mice relapsed, in some cases more than 3 months after chemotherapy. This was not due to drug resistance of the parasite, and tissue transfer experiments during the aparasitaemic period following chemotherapy indicated the presence of trypanosomes only in brain tissue.

Transmission of immunity from mother to offspring was investigated in mice. Mothers with a T. brucei infection conferred immunity to homologous challenge of approximately 3 weeks duration in newborn mice. This immunity was by way of colostrum/milk after birth, since fostering experiments showed that mice born of infected mothers but suckled by an uninfected mother were susceptible to challenge. Furthermore there was an augmentative effect when chemoprophylaxis was given on the day of birth. This on its own afforded protection against infection for a similar period as passively acquired antibody, but young mice which received both drug and antibody resisted challenge for over 6 weeks.

Genetic resistance to T. congolense infection was studied in C57Bl mice, which are able to repeatedly limit the numbers of circulating parasites and survive for approximately 80 days, and CFLP mice which die within 10 days with an uncontrolled fulminating parasitaemia. No evidence was found to implicate pathophysiological factors in this

resistance, but C57Bl mice were able to remove radiolabelled parasites from their circulation, and their immune response, as judged by *in vitro* immunological methods, was superior to the CFLP strain. Attempts to enhance the response of susceptible mice by passive immunisation, priming of the immune system and activation of the mononuclear phagocytic system were unsuccessful, and it was concluded that the ability to produce and maintain levels of IgM in the plasma was essential for resistance.

Finally, a reliable and simple technique for the *in vivo* labelling of trypanosomes with [^{75}Se]-methionine was developed. The fate of such trypanosomes after injection into normal and immune mice was investigated, and it was found that in immune mice the liver was the principal site of uptake. From this finding, studies were done on the mechanisms of hepatic uptake, which was found to be antibody dependent, and at low antibody titres also C3 dependent. No evidence was found to suggest that intravascular lysis or activated macrophages were involved in immune clearance.

GENERAL INTRODUCTION

The disease

Animal trypanosomiasis is one of the major constraints to the economic development of animal production in Africa. Because of the widespread distribution of the insect vector and the limitations of current control measures, it is estimated that 10 million square kilometres of tropical Africa, approximately 40% of the land mass (Buxton, 1955; Ford, 1975), suffer the consequences of the disease. Of all the livestock diseases endemic to the African continent, trypanosomiasis has been regarded as the biggest single factor which limits the numbers and productivity of cattle, sheep and goats (Urquhart, 1974). It is largely responsible for the inability to realise the enormous potential assets of savannah grazing in Africa, and in consequence contributes greatly to the serious nutritional and economic situation in these areas, and on a lesser scale also causes loss through morbidity and mortality of livestock in those areas which marginate fly belts.

Hornby (1952) stated "Trypanosomiasis is unique among diseases in that it is the only one which by itself has denied vast areas of land to all domestic animals other than poultry". As a social hazard its potential has also been stressed: "Sleeping sickness (African trypanosomiasis) constitutes a permanent and serious risk to the health and well-being of at least 35 million people, and animal trypanosomiasis is the main obstacle to the development of the vast potential for livestock development in the (African) continent" (WHO, 1976).

Lumsden (1968), discussing the economic importance of trypanosomiasis, detailed certain facts: the area in Africa virtually devoid of livestock from this cause is 10.4×10^6 sq km, and it was estimated in 1963 that this would be capable of supporting 125×10^6 cattle, which was considerably more than the total population in Africa at that time (Wilson, Morris, Lewis and Krog, 1963). Finelle (1974) estimated the consequences of trypanosomiasis control on cattle numbers, and by assuming a mean carrying capacity of 20 cattle per sq km in the 7 million sq km which he regarded as being tsetse infested, calculated a production potential of 140 million head if the disease were controlled, a seven-fold increase over the 20 million head quoted as the population at that time. Using a similar argument, but calculating from the tsetse infested area quoted by Lumsden (1968), Jasiorowski (1972) concluded that with trypanosomiasis control, 120 million cattle could be raised, assuming a mean carrying capacity of 12 head per sq km. He included in his estimate, however, 3 million sq km of equatorial rain forest which Finelle (1974) excluded as being unsuitable for livestock production. According to published figures, (FAO, 1976), the total cattle population of Africa in 1973 was 145 million, and in 1975 tsetse-infested regions were carrying 52.5 million head, but despite these discrepancies in numerical terms, the hypothetical estimates emphasise the importance of the disease in limiting livestock production and also how difficult it is to calculate its economic impact on Africa.

It has been stated that at least half the world's population is either undernourished or suffering from malnutrition

(Wright, 1961), and that the average daily intake of protein in underdeveloped countries is insufficient for normal growth and mental development (Jasiorowski, 1972). According to FAO, however (FAO, 1976), 37.5% of the world's livestock resources are located in underdeveloped countries, therefore it is not the number of animals which is responsible for the low level of animal protein production in these countries, but their lack of productivity.

Thus, it is clear that the benefits resulting from effective trypanosomiasis control lie not only in increased livestock numbers and a reduction in the mortality due to the disease, but also in improved carcass quality and increases in growth rate, milk production and fertility (Finelle, 1974).

The parasite

The causative agent of trypanosomiasis (or nagana as the cattle disease is called) which affects man, his domestic livestock, and wildlife are protozoan haemoflagellates comprising several species of the genus Trypanosoma. They are blood-borne parasites ranging from 8 to 39 μm (Mulligan, 1970), spindle shaped with a centrally placed oval nucleus, and they possess a single elongated mitochondrion which extends along much of the length of the cell. Towards the posterior end of the cell is a small, densely-staining structure known as the kinetoplast which contains DNA. In close proximity to the kinetoplast is a small pocket (flagellar pocket) from which emerges a single flagellum which is attached along the

longitudinal axis of the cell. The trypanosome also possesses a prominent layer of microtubules which lie just beneath the plasma membrane. The organisms are extremely motile by virtue of their flagellum and it is thought that the microtubules help to maintain cell shape during locomotion. Several of the structural features, such as the size and position of the kinetoplast and the presence or absence of a free flagellum at the anterior end of the organisms are used to distinguish the different species of trypanosomes in stained thin smears (Table 1). Perhaps the most important structural feature of the trypanosome, with regard to its ability to induce persistent infections in its mammalian host, is the possession of a surface coat. This cell coat, which is approximately 15 nm in depth and is only visible at the ultrastructural level, covers uniformly the entire plasma membrane and is composed predominantly of a single glycoprotein molecule of M.Wt. 55,000-65,000 K. It is this glycoprotein on the surface of the trypanosome which changes antigenically during the process of antigenic variation (Cross, 1978).

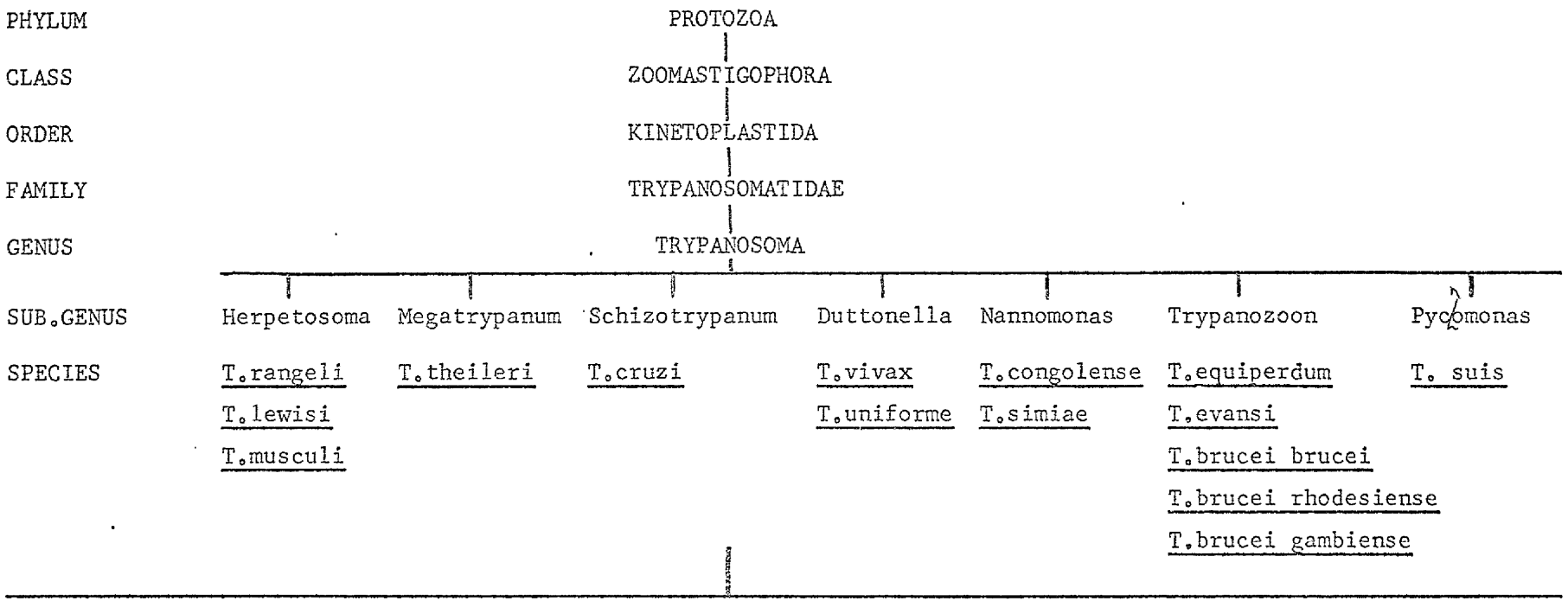
For practical purposes, Hoare (1972) proposed a classification restricted to those trypanosomes infecting mammals and therefore of medical and veterinary importance (Fig 1). He proposed two "sections" at a level between genus and subgenus: the Stercoraria, comprising species whose developmental cycle in the insect vector is typically completed in the rectum (or posterior station) and in which transmission is "contaminative", ie, by infective forms contained in the faeces of the vector

Table 1 Characteristics of trypanosomes pathogenic for cattle

Species	Site of development in tsetse fly	Bloodstream forms in cattle			
		Size (μm)	Morphology		Behaviour
T. congolense	Midgut	9-18	Kinetoplast	medium-size, marginal	Sluggish movement, often attached to red cells
			Posterior-end	blunt	
	Proboscis		Undulating membrane	poorly defined	
			Flagellum	no free flagellum	
T. vivax	Proboscis	20-27	Kinetoplast	large, terminal	Very rapid movement across the microscope field
			Posterior-end	rounded	
			Undulating membrane	usually poorly defined	
			Flagellum	free	
T. brucei	Midgut	15-39*	Kinetoplast	small, subterminal	Rapid movement in confined areas
			Posterior-end	pointed	
	Salivary glands		Undulating membrane	well-defined	
			Flagellum	free	

*Polymorphic - slender, intermediate and slender forms; no free flagellum on stumpy form.

Fig 1 The classification of trypanosomes



After Hoare, C.A. (1972)

invading the new host via skin abrasions or mucous membranes; and the Salivaria, those species whose developmental cycle is typically completed in the mouth parts (or anterior station) of the vector and whose transmission is "inoculative", ie. by infective forms being injected into the new host by a bite or in the saliva of the insect vector. Both sections are represented in Africa, although only the salivarian parasites are of medical and veterinary importance, causing sleeping sickness in humans and nagana in domestic animals.

No major disease is associated with Stercorarian parasites in Africa, although T. (Megatrypanum) theileri infections are frequent but usually asymptomatic and undetectable. However, Stercorarian parasites do cause serious problems elsewhere, 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.

Three distinct species, belonging to different sub-genera, are the major pathogens in cattle in Africa. These are T. (Nannomonas) congolense, T. (Duttonella) vivax and T.b. brucei which belongs to the subgenus Trypanozoon. All 3 species are found in cattle throughout the tsetse infested areas, and it is not uncommon to find all 3 trypanosomes in the same animal. The 3 species can be distinguished on morphological grounds and by characteristic behaviour when observed in a fresh wet blood film (Table 1).

T.b. brucei is closely related to and morphologically

indistinguishable from the human pathogens T.b. gambiense and T.b. rhodesiense which belong to the same subgenus. Unlike T.b. brucei these subspecies are not found throughout the tsetse belt but are restricted to loca lised foci. While the evidence as to whether or not T. gambiense can infect cattle is conflicting, infections with T. rhodesiense have been confirmed (B.T. Wellde, personal communication). In such situations, when trypanosomes with the morphological characteristics of T.b. brucei are isolated, the only certain way at present of distinguishing the subspecies is to ascertain whether or not the organisms are infective for man. Over the years these organisms have been regarded as separate subspecies, but more recently, it has been suggested that they belong to the same species and that through time T.b. brucei may give rise to variants which are infective for humans (Robertson and Pickens, 1975 ; D.D. Whitelaw, unpublished observation). It is known that human plasma can be cytotoxic for T.b. brucei, while certain T.b. rhodesiense and T.b. gambiense strains are resistant; thus the blood incubation infectivity test (BLIT) was devised in an attempt to distinguish between these subspecies (Rickman and Robson, 1970).

When examining blood samples from cattle in endemic areas of trypanosomiasis, T. congolense and T. vivax are usually found more frequently than T. brucei, and in general are considered to be more pathogenic. However, by subinoculation of blood into laboratory animals, a much higher incidence of infection with T. brucei may be found, and results of a number of studies indicate that T. brucei can

be pathogenic in its own right (Murray, Murray, Wallace, Morrison and McIntyre, 1979; Morrison, Murray, Sayer and Preston, 1981).

The African trypanosomes are cyclically transmitted by tsetse flies, genus Glossina (Mulligan, 1970), which become infected after feeding on an infected animal. The organism undergoes a number of developmental stages in the tsetse (trypomastigote, epimastigote, and metacyclic forms) before becoming infective once more for the mammalian host, there are marked morphological and structural alterations experienced by the trypanosome which occur within the fly, and for each trypanosome species there are differences in the sites in which some of the developmental stages occur (Table 1). The length of the life cycle within the tsetse also varies with each species.

Control of the disease

Since the precolonial days of African exploration when men such as Burton and Livingstone first brought the tsetse fly problem to the attention of the world, attempts have been made to control or eliminate the disease, with varying degrees of success. Phillips (1959) estimated that an expenditure over 50 years of one thousand million pounds would be required to eliminate the fly, and in the current economic climate this figure would have to be increased severalfold. Despite extensive control efforts this century, it was the opinion of Ford (1971) that "the area infested with tsetse in Africa is larger than it was in 1900 after the passage of the rinderpest".

The elimination of larger African mammals as a means of controlling trypanosomiasis has proved successful in the past. It was first advocated by Bruce, but the game slaughtering campaign between 1930 and 1950 in Rhodesia was based not on Bruce's thesis but on the historical evidence of the Rinderpest panzootic (1895-96) and the virtual extermination of the fly's favoured hosts. Ten thousand sq miles in Rhodesia were reclaimed from Glossina morsitans by game slaughter, land which had been reinfected following the recovery of the wild animal population after the rinderpest outbreak (Chorley, 1958; Cockbill, 1960). Despite this success however, the effectiveness of such control methods is limited due to the rapid recovery of game populations after culling (Percival, 1918) and the attendant fly reappearance. Unless game eradication is repeated, for the method provokes powerful opposition (Ford, 1970), its effect is transient, and despite its success in particular locations (Bursell, 1960), extensive use in the future is unlikely (Harley, 1978).

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 procedure 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. Similarly, the culling of wildlife must be done to maintain the integrity of the corridor, as these animals are regarded as an important reservoir of the disease.

Several interesting alternatives have been employed in recent years, including the use of the sterile male technique (Dame, 1970). However, one major constraint on 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. Thus, a considerable amount of the available resources have to be diverted into the maintenance of boundaries by insecticide spraying.

Total freedom from the disease is not a guaranteed consequence of an effective vector control campaign, as mechanical transmission is known to occur in the field (Baker, 1968) and has been demonstrated experimentally (Duke, Mettam and Wallace, 1934; Heisch, 1952). The influence that this has on the epidemiology of human and animal trypanosomiasis is unknown, but it is certain that in the absence of the tsetse fly the disease would remain a problem. Indeed, continued mechanical passage can lead to increasing virulence (Mesnil, 1912), and in pleomorphic strains there is also a tendency for them to become monomorphic (Oehler,

1914). This is typified by T. evansi which is thought to have arisen from T. brucei (Hoare, 1972) but is now virtually completely 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.

Arsenic compounds were used as early ago as the mid 19th century to combat nagana in Africa. However, it was recognised at the beginning of this century that some drugs produced as a by-product of the German dye industry had trypanocidal activity. Thus, research was stimulated by the discovery that Trypan Red was both curative and prophylactic in experimental trypanosomiasis and had potential in the treatment of the human disease. Further advances led to the development of Atoxyl, an organic arsenical derived as a by-product of the 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).

Trials using Tryparsamide were commenced in 1920 in a campaign against human sleeping sickness in the Belgian Congo, and 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 in Southern Rhodesia against animal trypanosomiasis with great success, and despite the discovery of other related compounds such as Antimosan, tartar emetic continued to be used against cattle infections on a large scale for many years (Jussiant, 1948).

In contrast to the measures against sleeping sickness in humans, chemotherapeutic and chemoprophylactic agents against trypanosomiasis in animals were not developed until relatively recently. As a result of the large range of species of animals susceptible to trypanosomiasis, the multiplicity of strains of trypanosomes and the different pathogenicity of the individual species, the problems involved in the chemotherapy and chemoprophylaxis of animal trypanosomiasis are more difficult, more complex and more serious than those involved in human trypanosomiasis. The few trypanocides which have been used in practice for a significant period beyond the testing stage are tartar emetic, antrycide, the phenanthridine derivatives ethidium, prothidium and samorin (isometamidium), as well as berenil and suramin.

In general terms, the most commonly used trypanocides in use at present are Berenil (diminazene aceturate), Samorin (isometamidium) and Ethidium (homidium chloride). Except in the case of long-standing infections, there is usually a good response to treatment: parasites are rapidly cleared from the blood and this is accompanied by a return to normal haematological values. However in endemic areas, reinfection can occur after treatment, depending on the degree of challenge.

In endemic tsetse areas where cattle populations are reasonably stable and animals can be easily identified by careful ear-tagging, chemoprophylaxis is a preferable approach to the control of trypanosomiasis. Thus, at present, cattle exist profitably on ranches which otherwise could not support cattle (eg. Wilson, Paris, Luckins, Dar and Gray, 1976). Samorin is the drug of choice, and in areas of constant heavy challenge, treatment may be required every 6 to 8 weeks. Less rigorous regimes can be worked out only after careful monitoring of each situation, but this approach utilising efficiently operated chemoprophylaxis should be considered in areas where eradication of tsetse is not feasible.

The principal problem associated with long term chemotherapy and chemoprophylaxis is the generation of drug-resistant parasites (Whiteside, 1963; MacLennan and Jones-Davies, 1967), but this can be minimised by the alternate use of different drugs and also by proper administration of drugs at the correct dosage levels. Unfortunately, Berenil has been the only new trypanocide to be made commercially available since the late 1950's, and in the event of the current drugs becoming ineffective, it is extremely doubtful that a new compound would become quickly available, since the demand for these drugs is limited and the potential profit too low to warrant expensive research and development.

Immunity to African trypanosomes

A typical trypanosome infection comprises a series of rising parasitaemias between which are periods 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.

This antigenic variation is now considered to be one of the crucially important mechanisms involved in the parasite's escape from the host's 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. 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, 1970). Whether secretion of the surface coat does occur during an infection, however, is still in dispute.

The ability to produce an immune response against a trypanosome infection has been regularly demonstrated either by infection followed by drug treatment (Bevan, 1936; Fulton and Lourie, 1945), vaccination with exoantigen (Herbert and MacAdam, 1971; Duxbury, Sadun, Schoenbechler and Stroupe, 1974) or vaccination with radioattenuated trypanosomes (Duxbury 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 and 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 and 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 but there are a few reports of the recurrence of certain variants within a few weeks of one another (Wilson and Cunningham, 1972; Nantulya, Doyle and Jenni, 1980). A clone of *T. equiperdum* has been shown to generate 101 different variants (Capbern, Giroud, Baltz and Mattern, 1977). There also appears to be a degree of consistency in the order of appearance of different variable antigen types (VATs) during infection so that certain VATs tend to be found during the early stage of infection while others tend to be present later.

One defect in the armoury of the trypanosome during its cyclical transmission in the tsetse has been described. This is the tendency for trypanosomes to resort back to a basic antigenic type. Thus, there is now evidence that the antigenic composition of the

metacyclic trypanosomes extruded by the tsetse fly is always similar for a given serodeme (Gray, 1965, 1975; Cunningham, 1966; Jenni, 1977; Nantulya, Musoke, Barbet and Roelants, 1979), even though it probably consists of a mixture of VATs (Barry and Hajduk, 1979), and the possibility that the number of serodemes may be limited in some location may offer the hope that immunization with metacyclic trypanosomes may be feasible.

In recent years, considerable progress has been made in the biochemical characterization of the variable antigen. Although initial studies, based on serological cross-reactivity and amino acid sequencing of the N-terminal end of the molecule, failed to demonstrate any homology between different variant antigens, more recent investigations have demonstrated the existence of cross-reactive determinants in variant surface antigen both within and between trypanosome species and the possibility of common structural regions has been suggested (Barbet and McGuire, 1978). However, it may be that these "common regions" are not exposed on the surface of the living organism. It is still uncertain whether the trypanosome possesses the genetic information necessary to code for the complete VAT repertoire or whether mutation or reassortment of genetic material occurs each time the antigen changes. However, in view of the marked heterogeneity between different variant antigens and the finding of some consistency in the order of appearance of different VATs, the former possibility appears more likely. The question still remains as to what triggers the change in surface antigen. While the disappearance of a particular VAT is associated

with the appearance of specific antibody in the serum, it is still uncertain if this acts as the primary stimulus for the surviving trypanosomes to change their variable antigen.

Protective immunity against a particular 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 of the parasitaemia has been further demonstrated by the use of various immunosuppressive regimes (Luckins, 1972; Balber, 1972; Ashcroft, 1957; vide infra) which showed 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 in these studies may not only have interfered with B cell responses but probably also 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 the earlier findings (Vickerman, Sless, Haston and Edwards, 1977; vide infra). Furthermore, the success of passive immunization using immune serum to confer protection strongly supports the importance of the humoral response (Seed and Gam, 1966; Patton, 1972; Takayanagi, Kambara and Enriques, 1973a; vide infra).

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 appear to be essential for the development of an immune response to trypanosome infection.

It is of interest to note that all of these previous studies were done either with T. lewisi or T. brucei-group parasites, and there is little comparable information on T. congolense infection (vide infra).

The effectiveness of the 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; Kobayashi and Tizard, 1976; Clarkson, 1976). The continually changing

antigenic stimulus during trypanosome infection presumably stimulates only the primary immune response, ie. 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 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 as being a better activator of the complement system than IgG. Trypanolysis may be therefore 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), but these authors also recorded the failure of immune mouse serum alone to initiate trypanolysis, presumably as a result of a deficiency in the complement system. Once removed from the host, mouse complement is extremely labile (I. McConnell, personal communication), thus 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 and Hildebrandt, 1974; Jarvinen and Dalmasso, 1976; Nielsen and Sheppard, 1976; Musoke and Barbet, 1977) and hypocomplementaemia has also been recorded as a feature of the disease. If an intact

complement system is essential for parasite removal, then hypo-complementaemia induced by the parasite may be an important mechanism by which the parasite can avoid trypanolysis (Nielsen and Sheppard, 1976; Musoke and 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 when it was shown that trypanosomes incubated in immune serum attached to platelets and monocytes (Nelson, 1953, 1963).

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 and Benacerraf, 1966; Mantovani, Rabinovitch and Nussenzweig, 1972; Bianco, Griffin and Silverstein, 1975; Hopf, Meyer zum Buschenfelde and 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 and Benacerraf, 1968) may be opsonic antibodies which act via attachment to the effector rather than the target cells, although Tizard (1969) has

described in mice both cytophilic and opsonic antibodies 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 the F $(ab')_2$ fragment of IgG, while the attachment to macrophages occurs via the Fc region of IgG (Takayanagi and Nakatake, 1977). It is therefore possible 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 and Enriques, 1974a).

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 and Mesnil, 1901). These workers observed that T. lewisi was phagocyosed in the peritoneal cavity of rats which had been actively or passively immunised. This was subsequently confirmed in vitro (Lange and Lysenko, 1960; Patton, 1972). A similar relationship has also been demonstrated in African trypanosomiasis (Lumsden and 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, 1974a).

The dependency of phagocytosis on antibody was recently confirmed by Takayanagi, Nakatake and 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) and may not therefore be an important impediment to attachment and phagocytosis, at least in antibody excess.

Despite these studies, it is not known whether whole opsonised trypanosomes (Lange and 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 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 and Cox, 1976; Clark, Cox and 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 and Clark, 1977). Since all 3 are readily detectable in trypanosome infected animals, increased hepatic uptake of SRBC in such animals (Murray, Jennings, Murray and Urquhart, 1974a) could well be a result of macrophage activation.

Evidence of resistance to trypanosomiasis in the field

Reports of several investigators showed no evidence of immunity developing in cattle maintained under chemotherapy in endemic areas over periods of time (Hornby, 1941; Wilson, Paris and Dar, 1975). Other workers, however, claim that drug therapy induced a degree of protective immunity in treated animals (Bevan, 1928; van Saceghem, 1938; Fiennes, 1953, 1970; Soltys, 1955; Smith, 1958; Wilson, Paris, Luckins, Dar and Gray, 1976; Bourn and Scott, 1978). These workers found that not only did the interval between infections increase as reflected by the frequency of chemotherapy, but also, when the decision to treat was made on the basis of detectable parasitaemia, animals were frequently parasitaemic without showing severe clinical effects.

Wilson reported a series of experiments which investigated acquired immunity. Cattle maintained in a high tsetse challenge area, treated on an individual basis either when clinically ill or when their PCV fell below 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 (Wilson, Paris and Dar, 1975). In contrast, when cattle were maintained in an area of medium tsetse challenge (Wilson et al, 1976), the interval between drug treatments increased while control cattle introduced at regular intervals became infected and required regular chemotherapy, thus indicating that the level of tsetse challenge may play a crucial role in the acquisition of resistance.

There are a number of possible explanations for the acquisition of resistance in the field. First, it is possible that cattle gradually develop specific immunity to all or most of the trypanosome serodemes in a particular location. Although each serodeme is capable of elaborating a large number of VATs during an infection, there is now evidence that the antigenic composition of the metacyclic trypanosomes extruded by the tsetse fly is always similar for a given serodeme (Jenni, 1977; Nantulya et al, 1979) and may be limited and consistent for each serodeme (Nantulya and Musoke, personal communication). Complete neutralisation of a metacyclic population of T. congolense can be achieved by serum taken from cattle 14-16 days after tsetse challenge (Morrison, unpublished data). Furthermore, animals treated with Berenil shortly after the establishment of infection are immune for up to 6 months to rechallenge with tsetse infected with the same serodeme, but are still susceptible to other serodemes. This has been confirmed in cattle with T. congolense and goats with T. brucei (Emery, Akol, Murray, Morrison and Moloo, 1980), although goats similarly treated after tsetse transmitted T. vivax infection showed no evidence of immunity (DeGee, 1980). Thus at least with T. congolense and T. brucei, if animals in endemic areas survive a primary infection with a particular serodeme, they should be able to resist subsequent tsetse challenge with that serodeme for at least 6 months. The possibility that the number of serodemes may be limited in some locations offers the hope that immunisation with metacyclic trypanosomes may be feasible.

Another factor which may contribute to acquired resistance in the field is antigenic cross-reactivity of VATs from different serodemes. Evidence of this has been obtained in studies with different repertoires of the sub-species Trypanozoon (Van Meirvenne, Janssens, Magnus, Lumsden and Herbert, 1975; Van Meirvenne et al, 1977). Some VATs derived from widely differing field isolates of T.b. brucei, T.b. rhodesiense, T.b. gambiense, T. evansi and T. equiperdum show strong cross-reactions in immunofluorescence and immune lysis tests with infection sera. These cross-reactive VATs have been termed iso-VATs, and although of similar molecular weight they have different isoelectric points. At present the frequency of these iso-VATs and their existence in T. congolense and T. vivax is uncertain, but if they are common, they might be expected to confer partial protection between different serodemes.

There is now evidence from serological studies that during a trypanosome infection certain VATs may recur within a few weeks of one another. This has been reported in cattle infected with a stabilate of T. congolense (Wilson and Cunningham, 1972) and a clone of T. brucei (Nantulya et al, 1980), but whether these represent identical VATs or iso-VATs is not known.

In addition to the cross-reactivity exhibited by iso-VATs, a glycopeptide at the C-terminal region of the variable surface glycoprotein (VSG) cross-reacts in all isolates and species of trypanosome so far tested (Barbet and McGuire, 1978; Cross, 1979; Barbet, McGuire, Musoke and Hirumi, 1979). However, unlike iso-VAT determinants, this common region is not accessible on the surface

of live trypanosomes and its role, if any, in acquired resistance is unknown.

The development of a significant resistance was observed in 3 adult Hereford cattle subjected to several needle challenges with unrelated stocks of T. congolense. Following successive challenges, each several months apart, the level of parasitaemia and the degree of anaemia which occurred were significantly less than in corresponding challenge controls. There was no evidence of serological cross-reactivity. An explanation for these findings, and possibly also a factor influencing acquired resistance in the field, came in further experiments investigating the effect of an established infection upon the susceptibility of the host to subsequent challenge. The results indicated that the initial infection interfered with the establishment of the second infection despite the absence of detectable antibodies against the trypanosome used for the second challenge (Murray, Morrison and Whitelaw, in press). This interference required the presence of an active infection, since animals were completely susceptible to the second challenge following treatment with Berenil, and may reflect some form of competition between trypanosome populations or a non-specific response of the host that limits the growth of trypanosomes.

It has been postulated that age appears to play a significant role in resistance to trypanosomiasis, young animals being more resistant than adults (Fiennes, 1970). This was thought by Whiteside (1962) to be through transmission of an immune factor

to calves born of immune or partially immune dams, but interpretation of much of the field data is difficult because of the use of small numbers of animals of unknown condition and the question of persisting trypanocides. However, one report showed significant age-related resistance in a large number of buffalo (Drager and Mehlitz, 1978), in which maternally derived immunity may have been an influencing factor.

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. The majority of published information suggests that trypanotolerant livestock are confined to West and Central Africa, but while this is broadly the case, there is no obvious reason why they should not have evolved in other regions of Africa infested by the tsetse fly. Indeed, Cunningham (1966) pointed out that thousands of Zebu cattle survive around the shores of Lake Victoria, even though they are continually exposed to tsetse, thus by definition they must be regarded as trypanotolerant. Similarly, certain breeds of sheep and goat in East Africa have a greater capacity to withstand the disease (Griffin and Allonby, 1979).

In one of the first accounts of West African livestock, Pierre (1906) recorded the ability of certain cattle to survive in tsetse-infested areas. Subsequently, the resistance of the taurine breeds was recognised increasingly in West Africa (reviewed by Godfrey, Leach and Killick-Kendrick, 1964 and ILCA, 1979), as

well as in the Sudan (Archibald, 1927). In 1951, Stewart described his experience with what he termed "West African Shorthorn Cattle" in Ghana (Gold Coast) over a period of 20 years from 1929 to 1948. These animals were genetically heterogeneous and comprised Hamitic longhorn, Shorthorns and Zebu. The contribution made by each 'breed' appeared to depend on the level of tsetse challenge; the greater the tsetse risk, the smaller percentage Zebu in each animal. His overall conclusion was that these animals possessed very high resistance to the disease.

The major fact by which trypanotolerance may be defined is based on the field observation that certain breeds of cattle, sheep and goats, as well as some species of wild animals, can survive in endemic tsetse fly-infested areas without the aid of chemotherapy where other breeds cannot. Most studies have focused on the N'Dama which is the main descendent of the Hamatic longhorn in Africa, and now the most common trypanotolerant breed in West Africa with an estimated population of 3.4 million head. Its popularity is probably related to the fact that it is the largest of the trypanotolerant breeds and is thought to be the most productive.

The superior resistance of the N'Dama to trypanosomiasis was consistently confirmed as judged by comparisons of weight loss, anaemia and survival. This was true regardless of the nature of the challenge. In some experiments, animals were exposed to natural field challenge of G. palpalis or G. morsitans (Chandler, 1952; van Hoeve, 1972), in others the animals were challenged with

G. palpalis infected in the laboratory with T. vivax (Chandler, 1958; Desowitz, 1959), while other investigations involved the inoculation of bloodstream forms of T. congolense (Chandler, 1958). These studies emphasised the potential of trypanotolerant livestock and presented evidence that trypanotolerance was an innate characteristic (Chandler, 1952, 1958).

While there is no question of the general validity of the foregoing results, some of the conclusions might be criticised for the following reasons; the clinical history of many of the animals was not precisely known, the antigenic characteristics of the various trypanosomes to which the animals were exposed was not investigated and, lastly, only small numbers of cattle were available for study. Without these data, it is impossible to define the relative contributions of innate and acquired resistance.

As a result, Stephen (1966) and Roberts and Gray (1972) evaluated the resistance to trypanosomiasis of N'Dama and Zebu which had been reared free from tsetse exposure. The Zebu dams had never been exposed to tsetse although the N'Dama dams had. Later, Roberts and Gray (1973a) extended these investigations to include N'Dama born of dams which had never been exposed to tsetse. In all these experiments the animals were challenged under laboratory conditions with wild caught G. morsitans submorsitans which were found to be infected with T. vivax, T. congolense and T. brucei. While once again the number of animals available was small and in some experiments there was a considerable age range (Roberts and Gray, 1972), the greater resistance of the N'Dama was obvious.

This result, assessed on the basis of weight loss, anaemia and survival, confirmed that the trait was an innate characteristic.

Susceptibility studies have also been done on the Muturu (a West African Shorthorn) breed of cattle, whose trypanotolerant characteristics were emphasised by Ferguson (1967). However, it was not until the studies of Roberts and Gray (1972, 1973a,b) that attempts were made to evaluate the extent of trypanotolerance in Muturu and compare it with N'Dama and Zebu. Their level of resistance was found to be intermediate between the N'Dama and Zebu, as judged by clinical condition, anaemia and survival. This result was confirmed by van Hoeve (1972) when animals of the same breeds were exposed to natural tsetse challenge in the field, and the superior resistance of the Muturu over the Zebu was demonstrated after needle challenge with T. vivax (Esuoroso, 1977).

The observation by Ssenyonga (1974) that C57Bl mice survived a T. congolense infection for a much longer period than CBA mice provided the opportunity for susceptibility studies to be done in the laboratory with animals of defined genetic background. This is the basis of the third chapter of this thesis (Chapter 3).

Secondly, the influence of maternal immunity has been investigated, in particular its role on early post-natal resistance

to trypanosomiasis and the effect on this of concomittant chemotherapy. This is the subject of Chapter 2.

The opening chapter describes studies on a feature of trypanosomiasis common to both animal and human infections, namely the problems associated with chemotherapy of chronic infections.

Chapter 4 details the development of a method for labelling trypanosomes with the amino acid analogue ^{75}Selenomethionine, and its use in studying the efficiency of the immune response in removing circulating trypanosomes. The relative importance of antibody, complement and the mononuclear phagocytic system in this removal is described.

Finally, parasite-induced immunosuppression has been suggested to be of practical importance in the response of animals to vaccination against other disease-causing agents. A collaborative investigation with the ODM Veterinary Team in Ethiopia, was done in mice and cattle, into the effect of chemotherapy at the time of vaccination against louping-ill virus on the subsequent antibody response of infected animals to the louping-ill vaccine.

The results of this study are appended at the end of the thesis.

CHAPTER I

CHEMOTHERAPY.

Section 1

The relapse of infection after chemotherapy

INTRODUCTION

The chemotherapy of trypanosomiasis in man and animals is at present dependent upon a relatively small number of synthetic drugs, against most of which drug resistance has been reported to occur. No new drugs have been introduced in recent years. In the absence of a vaccine against the parasite, and considering the expense and problems inherent in an effective vector control compaign, the use of chemotherapeutic and chemoprophylactic drugs remains the only alternative in combating the infection in susceptible humans and livestock. However, a necessary requirement in the successful treatment of the disease is early diagnosis and adequate surveillance of treated individuals after chemotherapy, and in the geographical areas where human and animal populations are at risk such conditions seldom prevail. Thus frequent use or mis-use of the compounds available has increased the dangers of drug resistance.

Strains of trypanosomes resistant to the commonly used trypanocidal drugs have been documented frequently in the literature (Williamson, 1970; MacLennan, 1970) and it has been shown that trypanosomes which are resistant to certain drugs may retain this property after transmission through the tsetse fly (van Hoeve & Grainge, 1966; Gray and Roberts, 1971). Although these reports have largely concerned strains of *T. vivax* and *T. congolense*, resistant strains of *T. brucei* have been recorded (Walker & Watts, 1970). Mwambu and Mayende (1975) reported that

of 23 T. brucei sub-group stabilates (3 from game animals, 10 isolated from cattle and 10 isolated from Glossina fuscipes) which were passaged in mice, only 1 stabilate was completely susceptible to the drug Ethidium. With the other 22 stabilates, the infections in a proportion of the mice relapsed.

This chapter deals with a type of relapsing infection in mice treated with trypanocidal drugs which in some respects resembles drug resistance as outlined above, but is apparently due to another cause, since the infections were always completely susceptible to chemotherapy administered at an early stage of the infection. The second section of the chapter deals with investigations to localise the focus of the cryptic infection, and the implications of the phenomenon are discussed in relation to the use of drugs in the natural disease.

MATERIALS & METHODS

Trypanosomes: Mouse passaged derivatives of stocks T. brucei TREU 667 and LUMP 1001 were used in most experiments. The histories of these parasites are outlined in Appendix I. T. brucei LUMP 227, 571, 612 and 962 were obtained through Professor W.H.R. Lumsden from the stabilate bank of the London School of Tropical Medicine and Hygiene, Winch's Farm, St. Albans, Herts., and were studied in one comparative experiment only. The T. congolense parasite used was designated GVR 1, and was derived in irradiated mice from T. congolense GUP 93. Its background is also outlined in Appendix I. The original stabilates were passaged in mice irradiated at 500 rad the day before infection, the blood removed on the ascending parasitaemia prior to the first peak, and preserved in polythene tubing in liquid nitrogen (Taylor, 1972).

T. brucei TREU 667/56 was isolated from a mouse which had been treated with Berenil after it had been infected for a period of 56 days with the original TREU 667/1: the infection recurred 30 days later, was transferred to irradiated mice and the frozen stabilate, TREU 667/56, was prepared after 4 days.

Clones of T. brucei TREU 667 were prepared by a modification of the method of Lumsden, Herbert and McNeillage (1973). The stock TREU 667 was grown in irradiated mice until the parasitaemia reached a level of approximately 1×10^7 per ml. The blood from the donor mice was diluted in phosphate saline glucose (PSG,

pH 7.2) containing 10% foetal calf serum (FCS) until a tiny drop on a glass coverslip could be expected to contain approximately 1 trypanosome. A hanging drop preparation was made by inverting the coverslip over a cavity microscope slide, the edges sealed with Vaseline to prevent evaporation of the drop, and examined under the microscope. The presence of a single organism in the drop was confirmed by a colleague before being termed a clone. The coverslip was then removed, the drop aspirated 3 or 4 times into a hypodermic syringe containing 0.2ml PSG with 10% FCS and injected intravenously into an irradiated mouse (500 rad). In this way, clones 667/1C, 667/2C and 667/3C were prepared.

Mice: All experiments with T. brucei species parasites were done in female CFLP mice (Anglia Laboratory Animals, England). At the start of experiments, these were 6 to 8 weeks of age and weighed approximately 25 g. Experiments with T. congolense GVR 1 were done in female inbred C57B1/6 mice (Laboratory Animal Centre, Edinburgh) of the same age. Mice were housed in either 5- or 8-capacity cages and were fed mouse diet pellets (Diet 41, Angus Milling Co., Perth) and water ad libitum.

Infection: Infections were initiated by intraperitoneal injection of 1×10^4 trypanosomes prepared from the frozen stabilates in phosphate saline glucose (PSG).

Monitoring of infection: Tail blood from the mice was examined for trypanosomes by the wet film technique prior to treatment to ensure that all were infected. After treatment, they were examined every two days for six days and thereafter twice per

week. In some instances, tail blood was inoculated into control mice to detect parasites. Precautions were taken to prevent accidental transmission of infection by removing positive mice from a group as soon as they became detectably parasitaemic. Permanent cure was considered to have occurred only if mice remained aparasitaemic for 200 days after treatment.

Drugs: The following drugs were inoculated intraperitoneally: Berenil (diminazene aceturate, Hoechst, Germany), Ethidium (homidium bromide) and Prothidium (pyrithidium bromide, Boots Pure Drug Company, England), Samorin (isometamidium), and Melarsen (N-(4,6 Diamino-5-triazin-2-yl)arsanilic acid monosodium salt heptahydrate (May and Baker Ltd., England). The doses selected for Berenil, Ethidium and Prothidium were several times greater than those recommended for cattle and were selected from experience to give maximum trypanocidal effect without evidence of toxicity in mice. Samorin was initially given at the dose level used for bovines, due to lack of previous experience of this drug in mice. Subsequently, the dose rate was increased to a level severalfold greater than its recommended dose. Melarsen was administered to reflect its use in the treatments of chronic infections in man. Accordingly, four doses were given on successive days, starting on day 21. The doses used for each drug are summarised in Table 1.1.

Immunosuppression of the host: This was done in 5 different ways. i) The powerful alkylating agent cyclophosphamide (Koch Light

Table 1.1 Recommended and Experimental Dosage of the Common Trypanocidal Drugs studied.

Drug*	Recommended dose mg/kg	Experimental dose mg/kg
Berenil**	3.5 - 7.0 AP	40.0 AP
Ethidium	1.0	7.5
Prothidium	0.2 - 0.4	10.0
Samorin	0.25 - 1.0	1.0 - 4.0
Melarsen	15	25

*Drugs were administered to mice on the basis of their weights to the nearest 5 g ie. a mouse weighing 23.5 g received a dose for a 25 g animal.

**The granule preparation of commercial Berenil is only 44.5% active principal. It was administered according to the following equation:

40 mg Active Principal/kg body wt.

= 90 mg granules / kg body wt.

= 2.25 mg granules/25 g mouse

∴ 22.5 mg granules is sufficient for 250 g body wt.

Dissolve 22.5 mg granules in 2.5 ml distilled water.

∴ 2.5 ml contains dose for 250 g body wt.

∴ 0.05 ml contains dose for 5 g body wt.

Thus, for a 20 g mouse, dose = 0.2 ml

25 g " " = 0.25 ml

30 g " " = 0.3 ml etc.

given at a dose of 300 mg/kg body weight, has a profound effect on the immune system of the host (Poulter & Turk, 1972; Lagrange, Mackaness & Miller, 1974), the effect being maximal 3 days after administration. Groups of mice were treated with cyclophosphamide (Cph) either 3 days before infection with T. brucei TREU 667/1 or on the same day as infection.

ii) Similarly, perturbation of host responses and enhanced parasitaemia result from treatment with β-methasone (Betsolan, Sigma, London). This was given on 5 consecutive days at a level of 1.0 mg/kg, starting the day after infection and ending 2 days after treatment.

iii) Sub-lethal total body irradiation at a dose of 650 rad depresses immune responses (Taliaferro & Taliaferro, 1964; Luckins, 1972). This was done the day before infection with trypanosomes, using a ^{60}Co irradiation source.

iv) Babesia microti infection. This piroplasm is a natural pathogen of mice, and is known to severely depress host immune responses concurrent with peak bloodstream parasitosis (Phillips and Wakelin , 1976). A stabilate of B. microti which had been cryopreserved in liquid nitrogen from an infected mouse was injected into splenectomised mice. When a 20% parasitosis was achieved the mouse was killed, and its blood used to initiate infection in three groups of eight CFLP mice. Patent parasitosis with Babesia occurred on day 5, reached a peak on day 10 and thereafter subsided. Trypanosome infection was initiated on day 9 and treated with Berenil on day 12. A horse red blood

cell (HRBC) haemagglutination assay was done in a control Babesia-infected group to assess the degree of immunosuppression induced by the parasite. 1×10^9 HRBC were injected on day 8 and the mice killed and serum collected on day 13. No antibody to HRBC was detected in the haemagglutination assay (Materials and Methods, p.128).

v) C57Bl mice were infected with T. congolense GVR1 for 21 days, superinfected with T. brucei TREU 667/1 and treated 3 days later with Berenil. The degree of immunosuppression exerted by the T. congolense infection was assessed as in iv) above.

The 5 different immunosuppressive protocols are summarised in Table 1.2.

Table 1.2 Experimental schedule for suppression of host responses prior to Berenil treatment of a 3 day infection with T. brucei 667/1

	(i)a	(i)b	(ii)	(iii)	(iv)	(v)
-21	-	-	-	-	-	T. congolense
- 9	-	-	-	-	B. microti	-
- 4	-	-	-	-	-	-
- 3	Cph	-	-	-	-	-
- 2	-	-	-	-	-	-
- 1	-	-	-	650 Rad	-	-
0	T.brucei	Cph + T.brucei	T.brucei	T. brucei	T. brucei	T. brucei
1	-	-	Betsolan	-	-	-
2	-	-	Betsolan	-	-	-
3	Berenil	Berenil	Betsolan + Berenil	Berenil	Berenil	Berenil
4	-	-	Betsolan	-	-	-
5	-	-	Betsolan	-	-	-

RESULTS

Horizontal spread of infection

The very nature of the experiments in this section necessitated mice being kept for long periods while being sampled for parasitaemia at regular intervals. Thus, the possibility existed that, despite meticulous cleaning of scissors with 70% alcohol after sampling each mouse in a cage, infection might be transmitted within a group through continued bleeding from the tail of an infected individual. Thus, others in a group may become infected horizontally by contact of their own open tail wounds. To test this possibility, 4 mice were infected with *T. brucei* TREU 667/1, and housed in a cage with 4 uninfected mice. All 8 mice were checked twice weekly for parasites by tail blood wet film. After the 4th day of infection, parasites were regularly found in the infected mice until between days 60 and 86, when these mice died of the infection. At no time were parasites found in the 4 control mice.

Time of treatment: The effect of time of treatment on the efficacy of eliminating *T. brucei* 667/1 in mice was tested by infecting a large group of 56 mice on day 0, and then removing from the original stock on days 3, 7, 14, 21, 28, 42 and 56, a group of 8 mice. These were isolated and treated with Berenil at 40 mg Active Principal/kg, and subsequently examined for a recurrence of parasitaemia as described.

The pattern of reappearance of parasites in the blood

after treatment is illustrated in Figure 1.1. The earliest reappearance was 21 days after drug treatment and the latest was 63 days after treatment, with the majority of breakdowns occurring between 20 and 50 days. There were no relapses if the mice were treated at 3 and 7 days after infection, but at 14 days, 6 out of 7 mice eventually relapsed. If administration of the drug was delayed later than this then all of the mice relapsed.

Treatment of relapse infection. To test if the relapse of the infection was due to the survival of drug resistant trypanosomes, a stabilate TREU 667/56 from a relapsed mouse (Materials and Methods) was inoculated into 2 groups of mice. The patterns of recrudescence of parasitaemia in this relapsed strain when treated 3 and 21 days after infection, together with that of the original 667/1 are shown in Figure 1.2.

The relapse strain of T. brucei 667/56, if treated 3 days after infection, was completely susceptible to Berenil treatment; however, if treatment was delayed until 21 days after infection then all of the mice showed a pattern of relapse similar to the original stabilate 667/1 ranging from 28 to 56 days after treatment.

Treatment of clones of T. brucei 667/1. To further substantiate that the survival of a few drug-resistant trypanosomes did not cause the relapse after Berenil treatment, three separate clones were prepared from the original 667/1. Each of these three cloned stabilates, 667/1C, 667/2C and 667/3C was used to infect 2 groups of mice which were treated with Berenil after 3 or 21 days. The

Fig 1.1

The reappearance of trypanosomes in the blood of individual mice given a single treatment with Berenil (40 mg/kg) on one occasion between 3 and 56 days after infection with Trypanosoma brucei 667/1.

 : Day on which trypanosomes first reappeared.

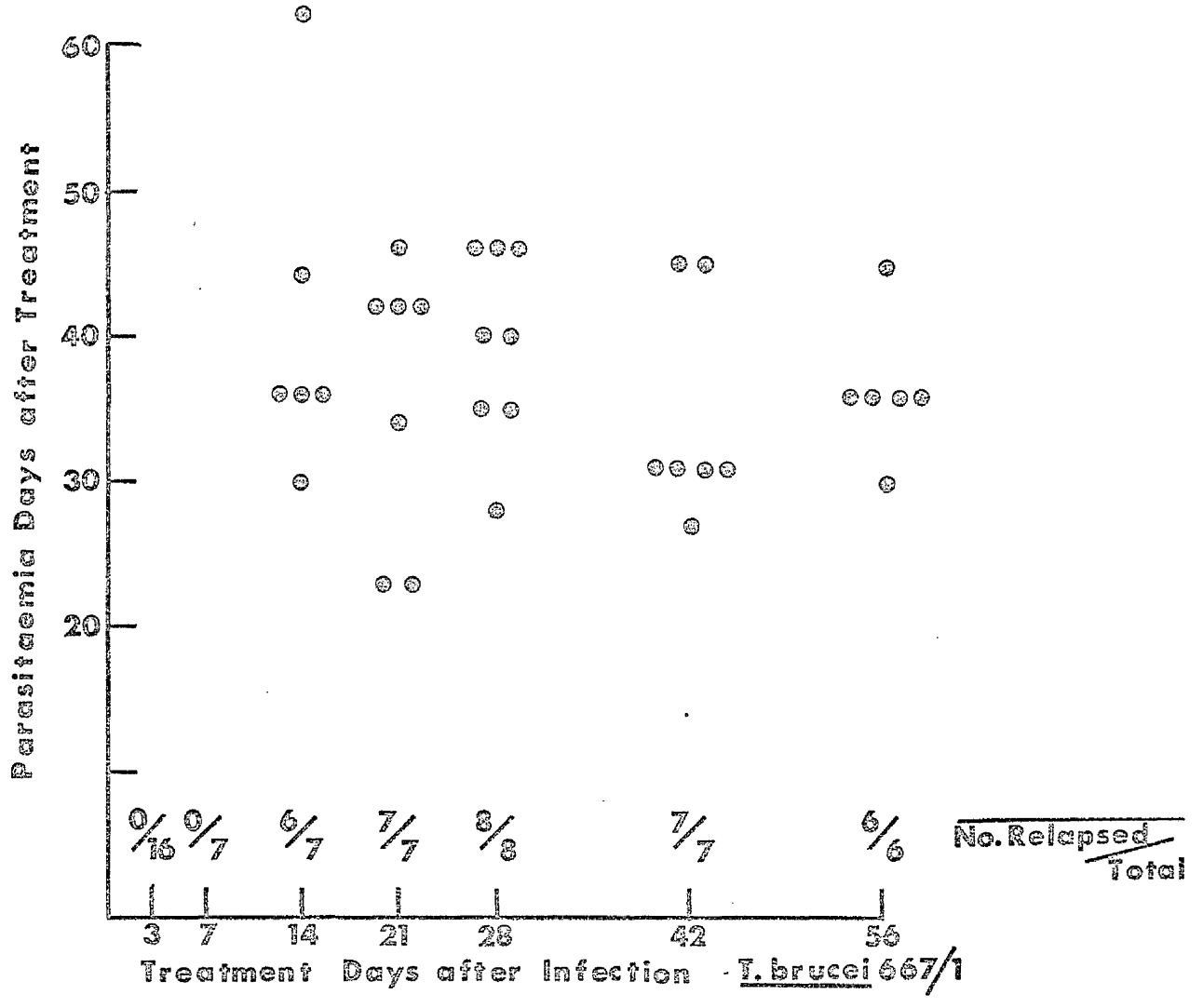
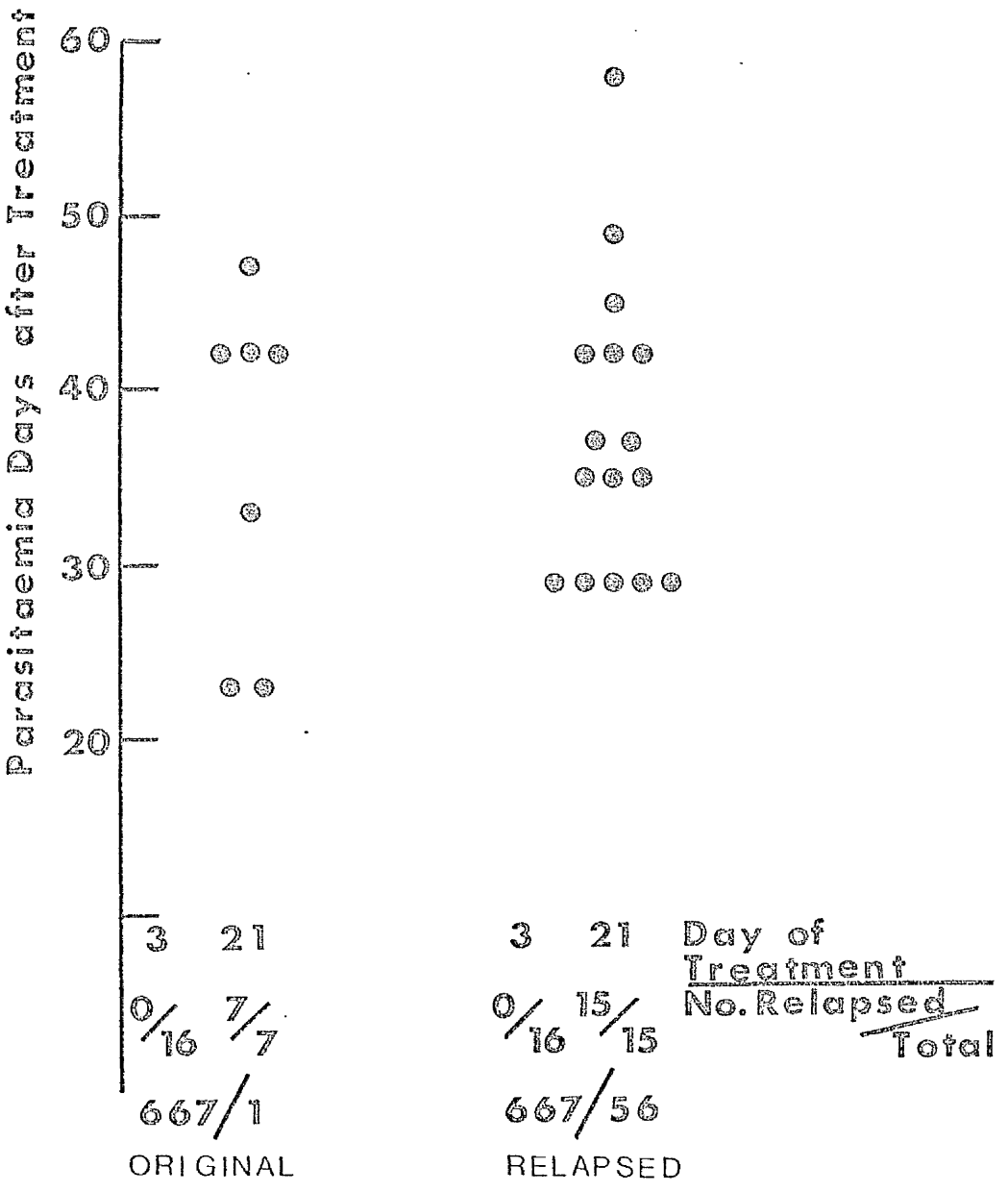

Parasitaemia Days after Treatment
60
50
40
30
20
0/16
0/7
6/7
7/7
8/8
7/7
6/6
No. Relapsed / Total
3
7
14
21
28
42
56
Treatment Days after Infection - T. brucei 667/1

Fig 1.2

The reappearance of trypanosomes in the blood of individual mice given Berenil (40 mg/kg) 3 or 21 days after infection with Trypanosoma brucei TREU 667/1, or TREU 667/56 isolated from a relapsed infection.

 : Day on which trypanosomes first reappeared.


Parasitaemia Days after Treatment
60
50
40
30
20
3
21
0/16
7/7
667/1
ORIGINAL
3
21
0/16
15/15
667/56
RELAPSED
Day of Treatment
No. Relapsed / Total

patterns of relapse shown in Figure 1.3, are essentially similar to the original 667/1 or the relapsed stabilate 667/56.

Effects of size of initial infection. In an attempt to show that relapses were not simply associated with a larger number of parasites being present when treatment was initiated late in the infection, 3 groups of mice received initial infections of 1×10^5, 1×10^6 or 1×10^8 T. brucei TREU 667/1, followed by treatment with Berenil at 3 or 21 days after infection. As before, all mice treated on day 3 were permanently cured regardless of infective dose. On visual appraisal most of the animals treated at 21 days had lower circulating parasitaemias than the 3-day infections, but the relapse patterns (Fig 1.4) showed no correlation with the size of the initial infections.

Effect of suppression of host responses. Immunosuppression of the host by cyclophosphamide or β-methasone treatment, total body irradiation, or B. microti or T. congolense infection did not influence the efficacy of chemotherapy. Treatment with Berenil after 3 days of a trypanosome infection resulted in permanent cure despite the immunosuppressive treatment.

Treatment of different stocks of T. brucei. In view of the results obtained with T. brucei TREU 667/1, the efficacy of Berenil was tested against the other strains of T. brucei available in the laboratory. Two stabilates, T. brucei LUMP 612 and LUMP 962 are particularly pathogenic for CFLP mice, and cause a high proportion of deaths by 3 weeks after infection. Treatment

Fig 1.3

The reappearance of trypanosomes in the blood of individual mice given a single treatment of Berenil (40 mg/kg) 3 or 21 days after infection with 3 cloned stabilates prepared from Trypanosoma brucei TREU 667/1.

⊛ : Day on which trypanosomes first reappeared.

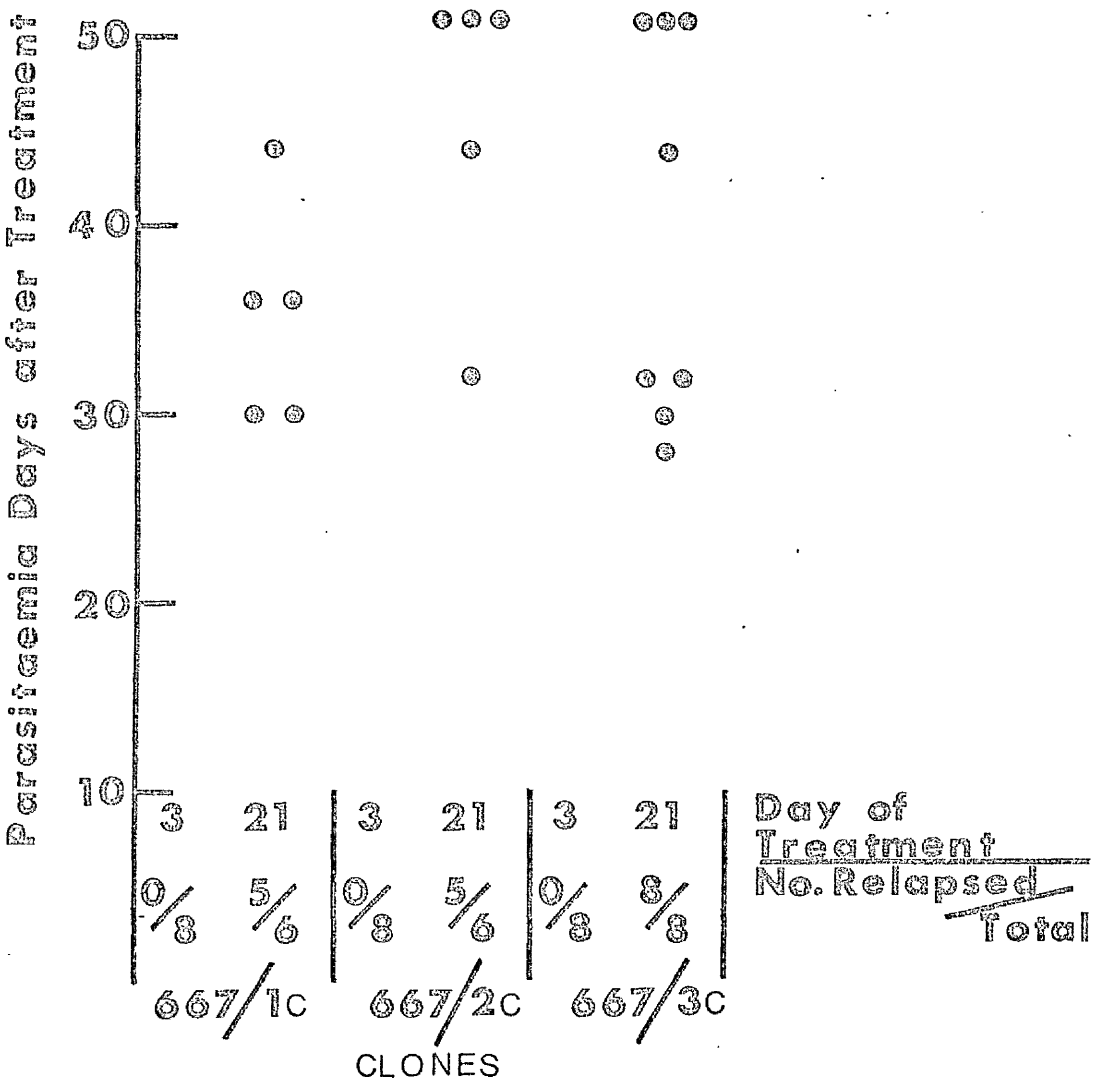
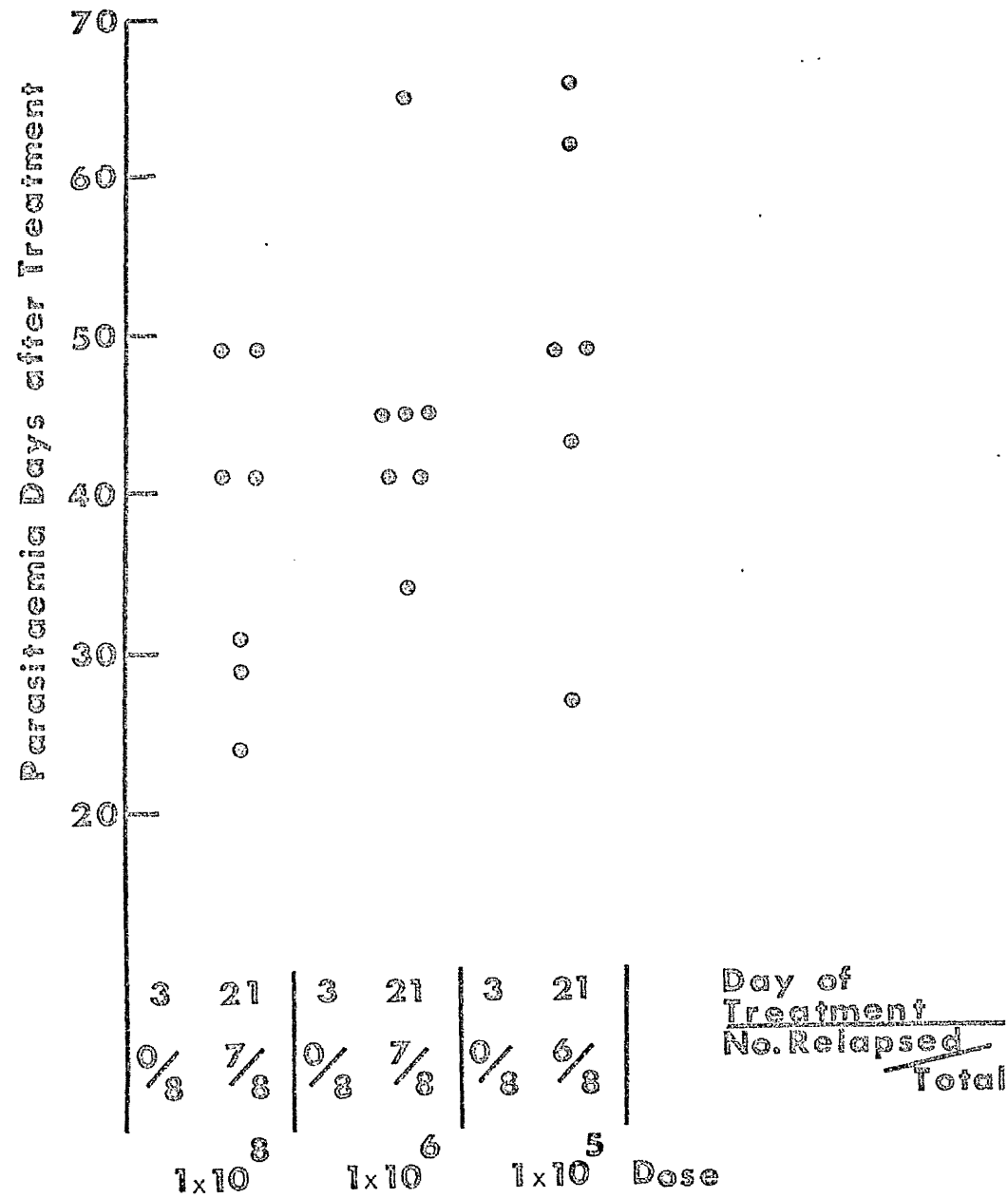

Parasitaemia Days after Treatment
50
40
30
20
10
3
21
3
21
3
21
Day of Treatment
No. Relapsed / Total
0/8
5/6
0/8
5/6
0/8
8/8
667/1C
667/2C
667/3C
CLONES

Fig 1.4

The reappearance of trypanosomes in the blood of individual mice given a single treatment of Berenil (40 mg/kg) 3 or 21 days after infection with doses of 10^5, 10^6, or 10^8 Trypanosoma brucei TREU 667/1.

● : Day on which trypanosomes first reappeared.


Parasitaemia Days after Treatment
70
60
50
40
30
20
3
21
3
21
3
21
0/8
7/8
0/8
7/8
0/8
6/8
Day of Treatment
No. Relapsed / Total
1x10^8
1x10^6
1x10^5
Dose

was therefore initiated on the day of the first fatality with these parasites ie. on days 14 and 12 respectively.

Of these other stabilates of T. brucei, only one, T. brucei LUMP 571, showed any indication of a relapse if treatment was at 3 days (1 of 8 mice); all the other mice treated at 3 days exhibited 100% susceptibility to Berenil (Fig 1.5), thus it is likely that the one relapse with T. brucei LUMP 571 was the result of a faulty injection. If treatment was delayed from between 12 and 21 days after infection, a high incidence of relapses was encountered with all stabilates.

Treatment with different trypanocidal drugs. The phenomenon of relapsing infections after chemotherapy was not confined to Berenil alone, but was also exhibited by Ethidium, Prothidium and Samorin (Fig 1.6). Ethidium and Prothidium were both 100% effective against T. brucei TREU 667/1 when treated at 3 days; however, if therapy was delayed for 21 days after infection, then Ethidium-treated mice relapsed 9-24 days later. Prothidium had, in general, a much more prolonged effect when given 21 days after infection, many of the mice remaining aparasitaemic until 60-120 days after treatment.

Samorin behaved somewhat differently from the other drugs in that at 1 mg/kg body weight, it failed to cure mice permanently. Even if treated at 3 days, 6 out of 8 mice became parasitaemic after approximately 30 days. However, this was probably due to drug underdosage, since in a subsequent experiment in which the

Fig 1.5

The reappearance of trypanosomes in the blood of individual mice given Berenil (40 mg/kg) 3 or 21 days (or at the time of first fatality) after infection with different stabilates of Trypanosoma brucei.

○ : Day on which trypanosomes first reappeared in a mouse treated 3 days after infection.

◍ : Day on which trypanosomes first reappeared in a mouse treated 21 days after infection.

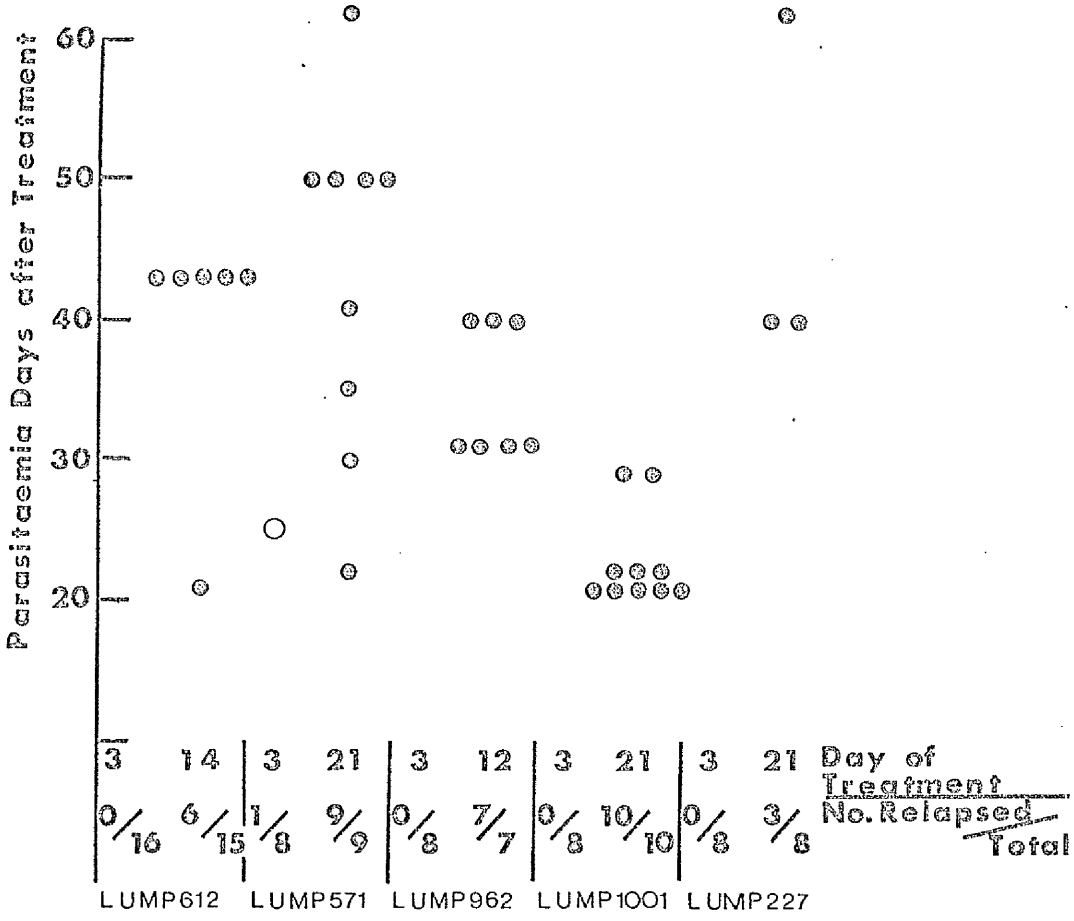
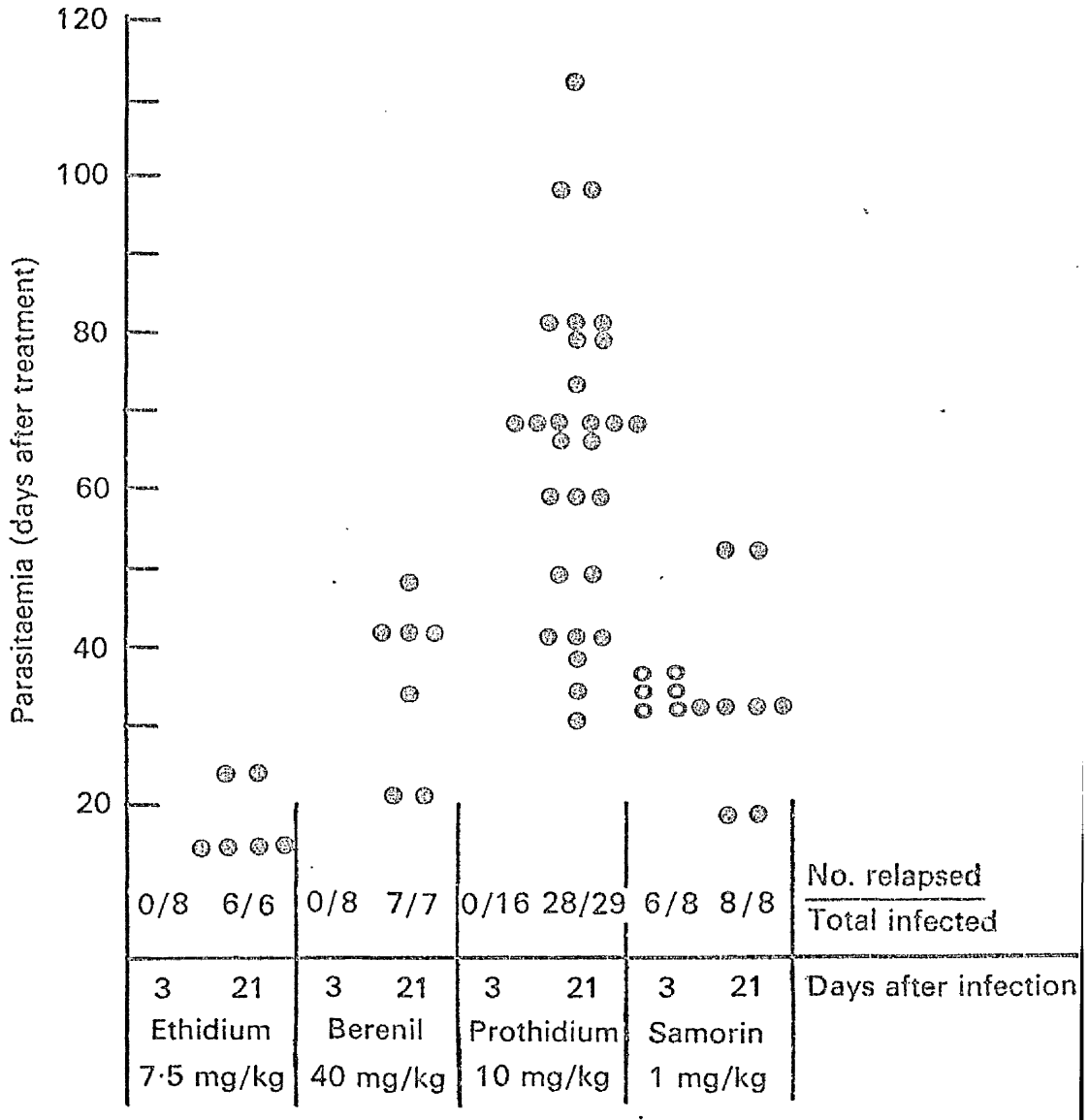

Parasitaemia Days after Treatment
60
50
40
30
20
3 14 3 21 3 12 3 21 3 21
Day of Treatment
No. Relapsed / Total
0/16 6/15 1/8 9/9 0/8 7/7 0/8 10/10 0/8 3/8
LUMP612 LUMP571 LUMP962 LUMP1001 LUMP227

Fig 1.6

The reappearance of trypanosomes in the blood of individual mice given a single treatment with one of 4 trypanocidal drugs 3 or 21 days after infection with Trypanosoma brucei TREU 667/1.

○ : Day on which trypanosomes first reappeared in a mouse treated 3 days after infection.

◍ : Day on which trypanosomes first reappeared in a mouse treated 21 days after infection.


120
100
80
60
40
20
Parasitaemia (days after treatment)
0/8 6/6 0/8 7/7 0/16 28/29 6/8 8/8
No. relapsed / Total infected
3 21 3 21 3 21 3 21
Days after infection
Ethidium 7·5 mg/kg
Berenil 40 mg/kg
Prothidium 10 mg/kg
Samorin 1 mg/kg

dose was increased to 4 mg/kg, permanent cures were obtained with treatment at 3 days, with substantial relapses occurring after a 21-day treatment even at this elevated dose (Table 1.3).

Effect of different doses of Berenil. The recommended livestock dose for Berenil is 3.5 mg/kg for T. congolense and T. vivax, and 7.0 mg/kg for T. brucei. At these doses, the drug is curative, and there is very little prophylaxis afforded by the drug. The Berenil dose in mice of 40 mg/kg was an arbitrary choice, being below the toxic level of the drug for mice, but at a level which effected elimination of the parasite for a considerable period of time. This was demonstrated in a dose-response experiment (Fig 1.7). Even at very high doses, permanent cures were not obtained in a large proportion of mice. At doses equivalent to the recommended livestock dose, permanent cure was obtained with treatment at 3 days, but such low doses were able to eliminate parasites from the bloodstream for only a short time when given on day 21 of an infection. 40 mg/kg was the dose at which parasites remained undetectable in the bloodstream after treatment for at least 4 weeks before recrudescence, and was below the threshold of drug toxicity.

Multiple treatment with Berenil. The effect of repeated treatments of Berenil on mice is shown in Table 1.4. Six groups of mice (groups A-F) were infected with T. brucei 667/1 on day 0 and all were treated with 40 mg/kg Berenil on day 21. Group A received only this one treatment, group B received this and a further treatment on day 42, group C was treated on days 21, 42

Table 1.3 The efficacy of Samorin and Melarsen on infections of *T. brucei* 667/1

Drug (dosage mg/kg)	Day(s) of treatment after infection	No. cured/ Total treated	Relapses occurred days after last treatment
Samorin			
(1.0)	3	10/10	
(1.0)	21	0/8	18-42
(2.0)	3	10/10	
(2.0)	21	0/10	35-71
(4.0)	3	10/10	
(4.0)	21	1/10	40-81
Melarsen			
(25)	21, 22, 23,24	0/20	10-18

Fig 1.7

The reappearance of trypanosomes in the blood of individual mice given different doses of Berenil 21 days after infection with Trypanosoma brucei TREU 667/1.

● : Day on which trypanosomes first reappeared.

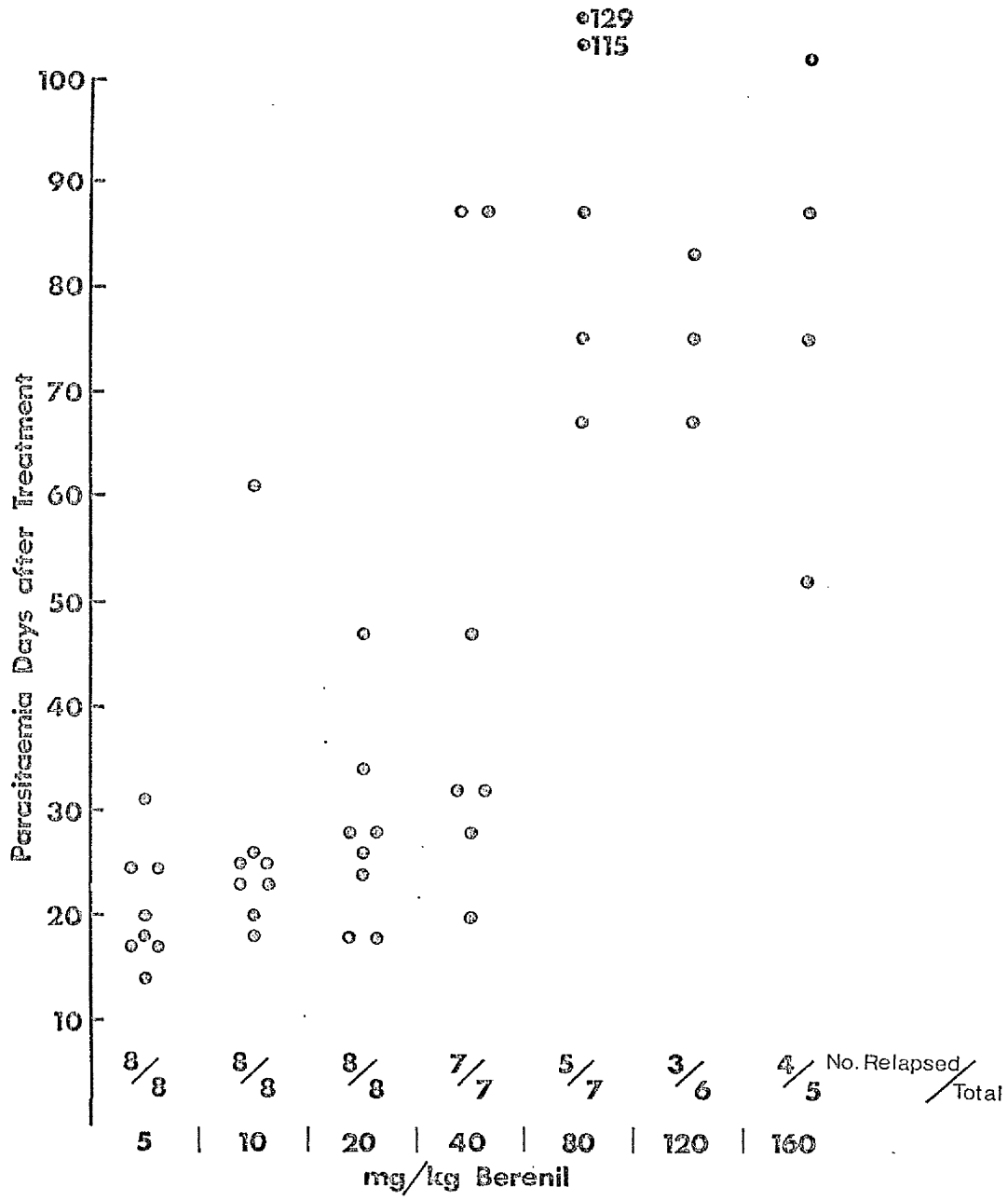

129
115
100
90
80
70
60
50
40
30
20
10
Parasitaemia Days after Treatment
8/8
8/8
8/8
7/7
5/7
3/6
4/5
No. Relapsed/Total
5
10
20
40
80
120
160
mg/kg Berenil

Table 1.4 The effect of repeated chemotherapy on T. brucei 667/1 infection

40 mg/kg Berenil	Day of treatment						No. cured/total treated	Day of relapse after last treatment
	21	42	63	84	105	126		
Group A	+	-	-	-	-	-	4/9	49 - 114
B	+	+	-	-	-	-	6/9	55, 98, 110
C	+	+	+	-	-	-	9/10	194
D	+	+	+	+	-	-	5/10	68 - 179
E	+	+	+	+	+	-	9/9	-
F	+	+	+	+	+	+	3/6	57, 62, 62

All groups were observed for over 200 days following the final Berenil treatment.

and 63, and so on. Thus, group F was treated on days 21, 42, 63, 84, 105, and 126. At no time were parasites detected in mice between treatments. With the exception of the group receiving 5 treatments, in which no relapses occurred, relapse of infection occurred after the cessation of treatment in all cases, and in some instances over 200 days had elapsed since the first treatment before parasites were detected in the bloodstream (Table 1.4).

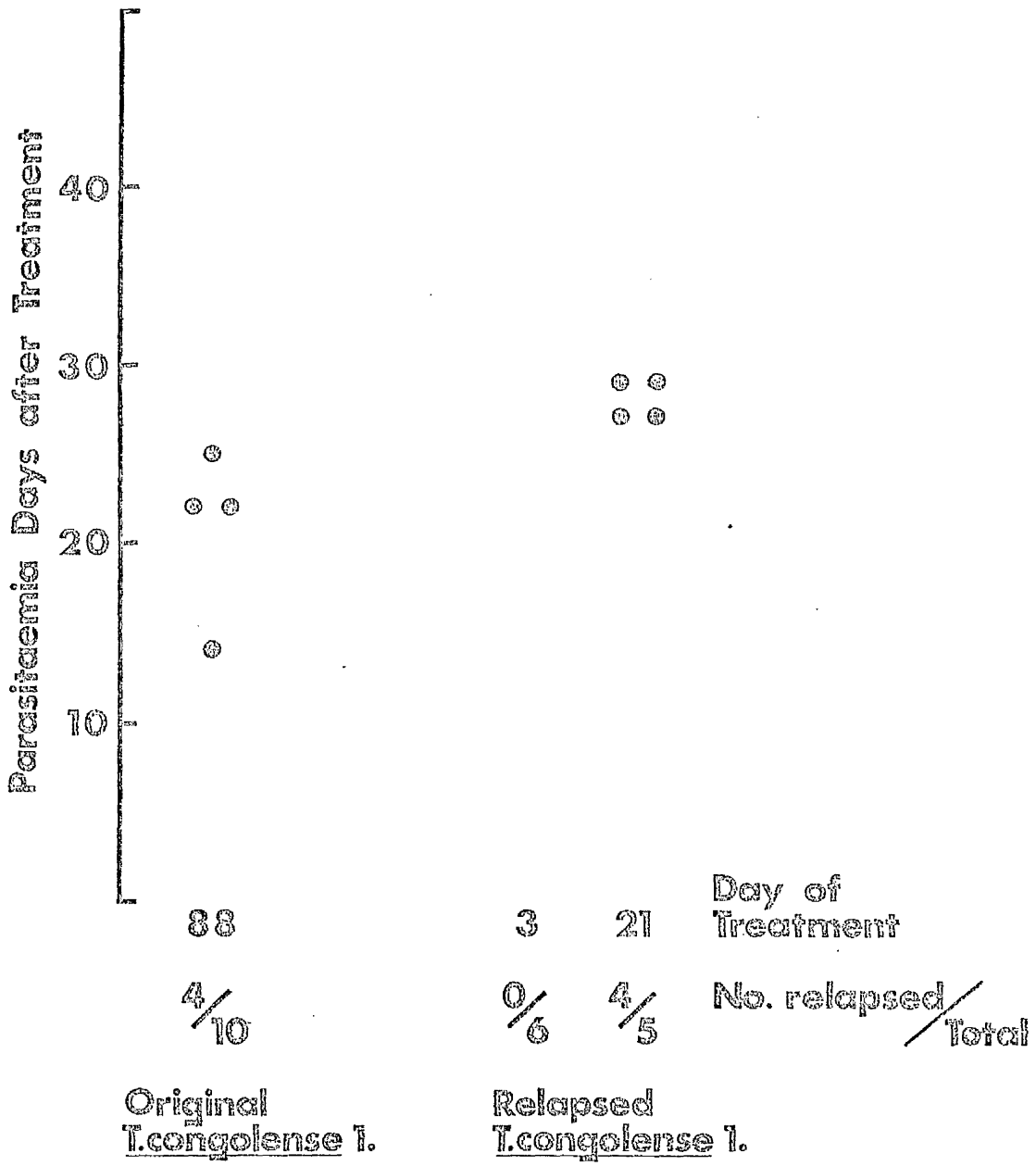
Relapse of T. congolense infection. Initial experiments with T. congolense infections in C57Bl mice did not show a similar outcome to those described for T. brucei. In these experiments Berenil chemotherapy at 40 mg/kg on day 21 elicited permanent cure.

To test if a longer period of time was required with T. congolense to create the conditions where relapse infections occurred, a group of 30 mice were infected with T. congolense GVR 1. By the 88th day of the infection, 10 mice still survived, and those were treated with 40 mg/kg Berenil. Four mice relapsed on days 14, 22, 22 and 25 after treatment. A stabilate was made from the blood of the mouse which relapsed on day 25, and 2 groups of mice were infected with this stabilate. One group was treated on day 3 and the other on day 21. Permanent cure was effected by treatment on day 3, whereas 4 out of 5 mice relapsed in the group treated on day 21, 2 on day 26 after chemotherapy and 2 on day 28 (Fig 1.8). The success of the Berenil therapy in these mice was confirmed by the inability to transmit infection with tail blood by inoculation into susceptible mice 7 days after treatment.

Fig 1.8

The reappearance of trypanosomes in the blood of individual mice given a single treatment of Berenil (40 mg/kg) 88 days after infection with T. congolense GVR 1, or 3 or 21 days after infection with a relapsed population.

● : Day on which trypanosomes first reappeared.


Parasitemia Days after Treatment
40
30
20
10
88
3
21
Day of Treatment
4/10
0/6
4/5
No. relapsed/Total
Original T.congolense 1.
Relapsed T.congolense 1.

A further experiment was done with this relapse population in which Berenil treatment was given at 20 mg/kg. Treatment on day 3 was completely successful, whereas trypanosomes reappeared in the bloodstream of 5 out of 7 mice on the 14th day following treatment on day 21. The relapse infections in these mice were allowed to continue for a further 21 days, at which time they were treated with 40 mg/kg Berenil. One mouse relapsed 18 days later. A stabilate was made of this population, and 3 groups of mice, groups A, B and C, infected, groups A and C with 10^4 parasites and group B with 10^7 parasites. Groups A and B were treated on day 3 with 40 mg/kg Berenil, and were permanently cured, whereas of the 8 mice in group C which were treated on day 21, 3 relapsed 22, 27 and 34 days later.

A summary of this experiment is shown in Fig 1.9.

Fig 1.9

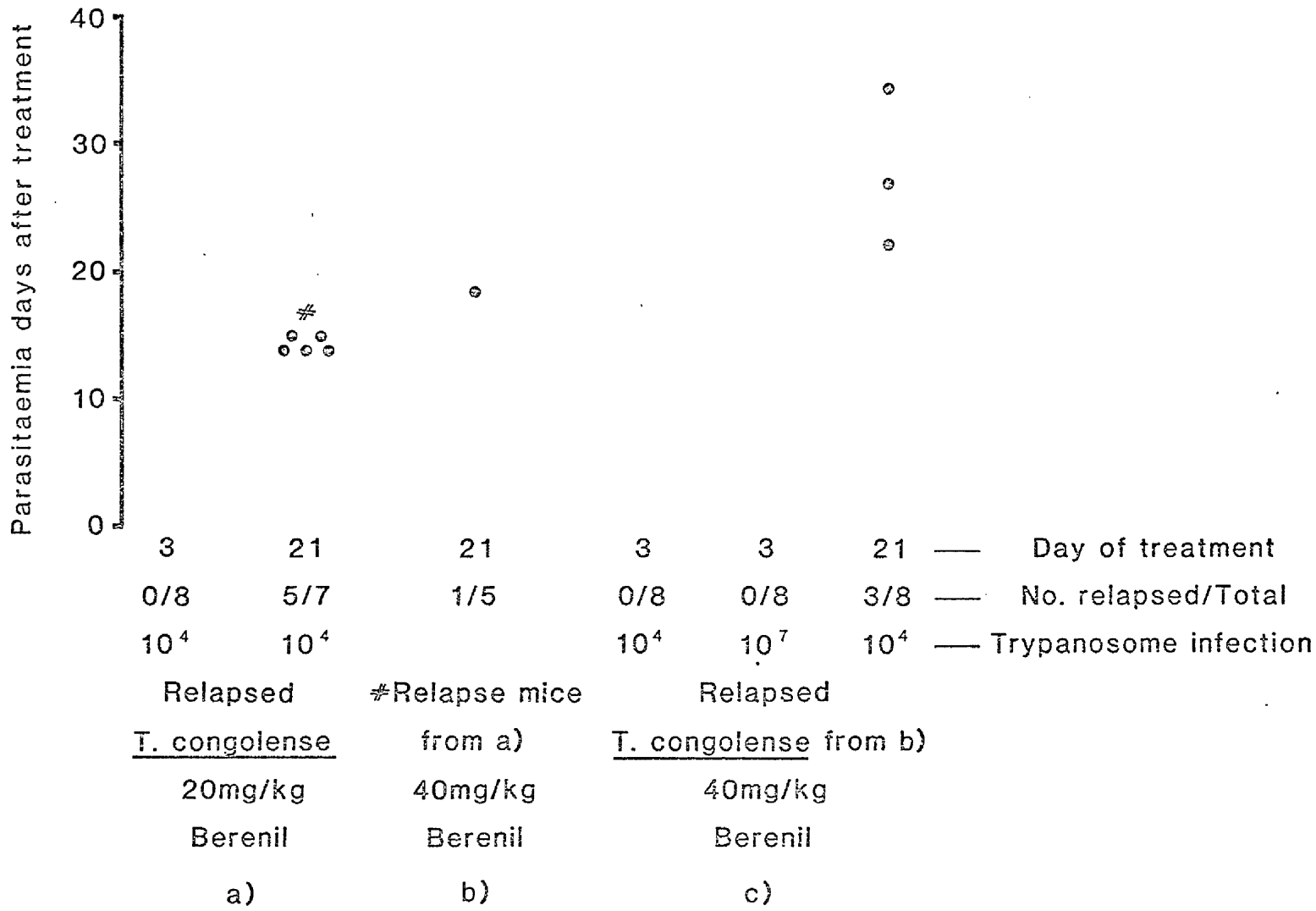
The reappearance of trypanosomes in the blood of individual mice given a single treatment with Berenil.

a) Treatment at day 3 or day 21 of relapsed T. congolense from Fig. 1.8 with 20mg/kg Berenil

b) 5 mice from a) which relapsed after 14 days were allowed to remain parasitaemic for a further 21 days and then treated with 40mg/kg Berenil

c) A stabilate was made from the one mouse which relapsed in b), and groups of mice were treated 3 or 21 days after infection with either 10^4 or 10^7 parasites.

⊗ : day on which trypanosomes first reappeared.


Parasitaemia days after treatment
40
30
20
10
0
3
21
21
3
3
21
Day of treatment
0/8
5/7
1/5
0/8
0/8
3/8
No. relapsed/Total
10⁴
10⁴
10⁴
10⁷
10⁴
Trypanosome infection
Relapsed
T. congolense
20mg/kg
Berenil
a)
#Relapse mice
from a)
40mg/kg
Berenil
b)
Relapsed
T. congolense from b)
40mg/kg
Berenil
c)

DISCUSSION

The experiments in this section have investigated the efficacy of chemotherapy in mice undergoing T. brucei and, to a lesser extent, T. congolense infections. From these studies, it is evident that relapses of infection after chemotherapy occur which can be ascribed neither to drug-underdosage nor drug resistance.

Extrapolation from a large animal system to a rodent experimental system is an exercise that must be done with extreme caution. Thus, a therapeutic dose of Berenil (40 mg/kg) many times greater than that recommended for infections in livestock was chosen to investigate the effect of chemotherapy on mouse infections. A similar approach was adopted with the other drugs. The justification for this approach was obtained in the Berenil dose-response experiment (Fig 1.7) which showed that at doses therapeutic for livestock infections (3.5-7.0 mg/kg), there is a very transient aparasitaemic period in mice after treatment before trypanosomes reappear in the blood. This experiment eliminated as a possible explanation for post-treatment relapse of infection the possibility of drug-underdosage. 40 mg/kg Berenil eliminated trypanosomes from the bloodstream for a considerable period of time during which they could be detected neither microscopically nor by subinoculation of blood into mice, and even at elevated doses which are toxic, relapse infections always occurred. For example, a dose of 160 mg/kg given as a single injection, or as 2

injections of 80 mg/kg given on successive days, or as 4 injections of 40 mg/kg over 4 days, failed to give permanent cure.

The complete efficacy of Berenil on a variety of stabilates of T. brucei if treatment was initiated 3 days after infection compared to an almost 100% incidence of relapses if treatment was delayed, suggests that the inability of chemotherapy to produce permanent cure is not due to drug resistance of the parasite. This is supported by the finding that a relapsed strain is completely susceptible to chemotherapy when treated at an early stage in a new host.

Further evidence against drug-resistance per se was the finding that 3 separate clones prepared from one of the original stabilates behaved in a similar manner after chemotherapy. Furthermore, no evidence was obtained that the incidence of relapses was influenced by the size of the initial infection.

The efficacy of early treatment raises the possibility of another explanation. It has been clearly established that T. brucei infection has a profound immunosuppressive effect on mice (Murray et al, 1974a,b)

Studies in cattle (Maxie and Losos, 1977) suggested that Berenil acted by making trypanosomes available to host defences such as the macrophage system, which eliminated them from the host. However, it is possible that through immunosuppression due to an established trypanosome

infection, the host's immune system cannot process drug-treated parasites, and as a result, subsequent relapse occurs. Thus treatment after only 3 days of infection is successful since the degree of immune impairment which is manifest by 21 days has not yet developed.

The results with irradiated, drug suppressed and Babesia microti-infected mice suggest that this is not the case. Thus, despite total body irradiation, treatment with cyclophosphamide or β-methazone, or a concurrent infection with another immunosuppressive parasite, permanent cure resulted in mice whose duration of trypanosome infection was only 3 days. While the interpretation of these results must be made with the proviso that the mechanism of immunosuppression induced by agents other than trypanosomes may be markedly different from that imposed by a trypanosome infection, the experiment in which a T. brucei infection was superimposed on a 21 day T. congolense infection supports the conclusion that immunosuppression is not involved in the relapsing phenomenon. Treatment on the 3rd day after challenge with T. brucei resulted in permanent cure.

An interesting observation in this experiment was made in a control group of mice superinfected with T. brucei, which was not treated with Berenil. On the 7th day following infection with 10^4 T. brucei 667/1 when peak parasitaemia would normally be attained in mice with a primary infection with this parasite, no T. brucei parasites were seen in Giemsa stained slides made from

tail blood of 3 out of the 10 mice in the group. All parasites displayed the typical morphology of T. congolense. This suggests that in some way establishment of the superinfection was inhibited by the existing T. congolense infection. Presumably, in the group treated on day 3 when the T. brucei infection was not yet patent, a similar preparation may have also failed to become established. However, within the context of this experiment, the remaining mice were permanently cured by chemotherapy.

The phenomenon of relapse after chemotherapy appears not to be a result of drug underdosage or drug resistance. Rather, it would seem to be a result of the trypanosomes escaping the action of the chemical, either because they are in an inaccessible situation in the body, or in an insusceptible stage of development. Either of these stages apparently requires some time to develop during the course of a normal infection in view of the susceptibility of early infections to drugs.

Furthermore, the evidence suggests that once either of the above states is reached, it can be maintained indefinitely, or the parasite can reattain such a state if required. Thus, the parasite can survive repeated treatments given over a prolonged period, yet still retain the capability of causing a relapse infection when treatment ceases.

This is similar in some respects to the observations of Browning and Calver (1943) and Calver (1945). However, in these experiments, cure was ultimately obtained in mice which showed

repeated relapses of a strain of T. congolense after repeated doses of a phenanthridinium drug.

It is not possible to account for the different lengths of time which elapsed between drug treatment and the reappearance of infection. With Ethidium and Berenil, the onset of relapse is relatively rapid and in the latter at least may be associated with the apparently rapid rate of excretion of the drug. Thus, Fink and Damm (1974) have reported successful infections of mice with T. congolense 5 hours after treatment with Berenil at 10 mg/kg, and Fairclough (1963) found that cattle were susceptible to experimental infections with T. congolense 24 hours after treatment at 3.5 mg/kg. However, other workers (van Hoeve and Cunningham, 1964; Lumsden, Herbert and Hardy, 1965; Raether, Hajdu, Seidenath and Damm, 1972; Zahalsky and Weinberg, 1976; Williamson, 1976) have shown that Berenil may have a prophylactic effect for up to 6 weeks after administration. These contradictory observations may be due to the use of different strains of T. brucei or depend on the numbers of trypanosomes used as the challenge inoculum; however, this will be further discussed in Chapter 2.

Most of the mice treated with Samorin or Prothidium (both of which are prophylactic drugs) did not show relapses until 40-70 days after treatment, and in some cases mice remained negative until nearly 120 days after treatment. The result from the second Samorin experiment suggested that, despite being used initially at the recommended livestock dose, such a level of Samorin is insufficient for the treatment of infections in mice.

Thus, the relapses obtained after treatment on day 3 with 1 mg/kg were undoubtedly due to drug underdosage, since higher doses resulted in permanent cure.

The result with Melarsen was interesting since a course with this drug has proved effective against the later stages of the Gambian disease in man. However, a dose of 25 mg/kg given daily for 4 days failed to cure a three-week infection in mice. The implications of this result will be discussed further in the 2nd section of this chapter. A possible explanation for the type of relapse described in these experiments could be the persistence of the amastigote phase of T. brucei infection described by Ormerod and Venkatesan (1970, 1971a,b). (The situation with T. congolense appears to be different and will be discussed separately.) These forms, primarily present in the choroid plexus, may be unsusceptible to drug treatment and produce fresh waves of bloodstream forms. In this connection, these authors (1971a) stated that "it is probable that all current trypanocidal compounds are active against the occult visceral phase, but this problem needs to be investigated specifically". However, this would not correlate with the findings of Soltys, Woo and Gillick (1969) and Soltys and Woo (1969, 1970), that tissue forms which would pass through a filter pore size 0.8 μm diameter could be detected in a number of organs as early as 72 hours after the administration of blood-form trypanosomes. Obviously, these forms, if present with these stabilates must still be drug sensitive, since treatment at 3 days would not otherwise give a permanent cure. There are also several reports (Schwetz, 1928;

Schwetz and Fornara, 1929; Fiennes, 1950a,b; MacLennan, 1973) in which the existence of an 'occult' or 'tissue' form of bovine trypanosomiasis caused by T. congolense and T. vivax was postulated, and Goodwin and Rollo (1955) have suggested that such forms might be relatively unsusceptible to the action of drugs.

Thus, if one assumes that re-emergence of bloodstream trypanosomes from drug-unsusceptible tissue forms does occur as postulated above, perhaps, as suggested by MacLennan (1971), even trace amounts of Berenil in the plasma are sufficient to kill these forms for a certain number of weeks. Eventually, however, the drug level falls below the level of trypanocidal activity and, if tissue forms still persist, parasitaemia will recur.

An alternative explanation is that the trypanosomes may be sequestered in a site in the host where they are inaccessible to the action of drugs. With such a situation, the capability to adopt a stage of development of the parasite which is unsusceptible to drug action is not required, and the trypanosome may merely exist in a sheltered environment and continue replicating as a bloodstream form. Provided that the integrity of the isolation mechanism remains intact and excludes trypanocidal drugs, one can visualise a situation where there may be continual repopulation of the bloodstream from this focus, which is eliminated as long as adequate drug-levels are maintained in the host. However, as soon as drug levels fall below that required for trypanocidal activity, the relapse infection is established.

These observations have a three-fold significance. First, if cattle exhibit a similar type of relapse phenomenon after treatment, much of the information on the epidemiology and immunity of the natural disease, which has been based on information obtained from the use of trypanocidal drugs, may require to be reassessed. This is particularly true if such relapses occur after treatment of T. vivax and T. congolense infections, and in this context, two reports from Nigeria are of interest. The first (MacLennan and Na'Isa, 1970; MacLennan, 1971) reports experiments conducted in cattle with a strain of T. vivax which regularly relapsed 10-25 days after treatment with Berenil at both normal and elevated doses. During the aparasitaemic periods no trypanosomes were detected in blood as tested by sub-inoculation into susceptible cattle and by the absorption elution technique of Lanham and Godfrey (1970). The second, by Gray and Roberts (1971) describes 2 strains of T. congolense and 2 strains of T. vivax which were resistant to therapeutic doses of trypanocidal drugs in that the infections generally relapsed 2-6 weeks after treatment. There was no loss of this drug resistance despite repeated transmission through tsetse flies. MacLennan (1971) administered Berenil on the 9th day after infection while Gray and Roberts, whenever possible, treated their cattle on the 5th to the 7th day of patent parasitaemia.

It is not possible to say whether or not these relapse infections in cattle are examples of true drug resistance or are due to the phenomenon recorded here in mice. However, it seems

desirable that the comparative incidence of relapse infections in cattle infected with T. congolense and T. vivax should be examined in relation to drug treatment administered very early and late in the course of infection. In this connection, a Zebu ox, which had been treated on day 21 of a T. congolense infection with 7.0 mg/kg Berenil, relapsed 69 days after chemotherapy (P.H. Holmes, personal communication). Since this experiment was done in a fly-free area of Ethiopia, there was no possibility of re-infection of the animal, and experiments in mice similar to those recorded have indicated that the relapse population was completely susceptible to treatment on day 3, eliminating drug resistance of the parasite as a possible explanation.

The experiments reported here indicated that although relapses of T. congolense infections occurred with less frequency than T. brucei, they nevertheless did occur, thus indicating further the influence that the phenomenon might exert in a field situation.

Secondly, the experimental evidence, at least in mice, that trypanosomes are more susceptible to drug treatment during the period of establishment in a new host is of importance in the evaluation of potential trypanocidal drugs, since it is very seldom that evidence for trypanocidal activity is sought in established infections (eg. Nathan, Soto, Moreira, Chunosoff, Hutner and Bacchi, 1979).

Finally, the chronicity of infection in man despite treatment, at least in T. gambiense infection (Apted, 1970) may be

associated with a similar phenomenon to that described here in mice.

The importance of T. brucei as a pathogen for domestic livestock has largely been underestimated because of the proven impact of T. congolense and T. vivax. Indeed, the literature cites many occasions where T. brucei has been stated as not being a major pathogenic species (Flennes, 1970; Killick-Kendrick, 1971), along with its related species T. rhodesiense and T. gambiense which are pathogenic for man. Moreover, recent studies have questioned the validity of separating T. brucei, T. rhodesiense and T. gambiense into subspecies in view of the ability of clones to produce human-infective and non-infective populations sequentially (N. Van Meirvenne, personal communication; DDW, unpublished). However, experimental studies (Murray et al, 1979; Wellde et al, 1980) have produced a fatal outcome in cattle with T. brucei and T. rhodesiense, and dogs (Morrison, Murray, Sayer and Preston, 1981) and goats (Emery and Moloo, 1980) are also very susceptible.

The experiments recorded in this section, if reproducible in livestock, raise the possibility of an animal being "infected for life" with trypanosomes, despite chemotherapy. The "cryptic focus" of infection which has been under discussion forms the basis for the second part of this chapter.

Section 2

The Brain as the Source of Infection after Chemotherapy

INTRODUCTION

Sequestration of a pathogenic organism in a site inaccessible to host defences and drugs, or in a stage of development such that normally effective drugs are unable to act, is a frequently observed phenomenon. Penicillin cannot eradicate tertiary syphilis in man, not because of the organisms becoming drug-resistant, but because of the presence of non-growing treponemes (Collart, Borel and Durel, 1962; Rice, Dunlop, Jones, Hare, King, Rodin, Mushin and Williamson, 1970). Furthermore, in experimental infection in rabbits, viable organisms persist for life and can be readily isolated from lymph nodes. It has been shown that hypobiotic fourth-stage Ostertagia larvae lodged in the gastric glands of calves, are insusceptible to many of the common anthelmintics (Anderson, Armour, Jarrett, Jennings, Ritchie and Urquhart, 1965). Brucella abortus may persist within mononuclear phagocytes (Karlstad, Kessel, de Petris and Monaco, 1964) where it is inaccessible to the action of streptomycin and host antibody, and even with the tetracyclines, eradication of some Brucella spp only follows prolonged treatment (Spink and Bradley, 1960; Phillipson, Kazmierczak, Plommet, Marly and Nevot, 1976).

Natural Leptospira infections in rats display chronic involvement of the kidneys, and similarly multiplicative infective parasites have been demonstrated in the kidney capillaries of mice which had apparently recovered from Trypanosoma musculi infection one year previously (Viens, Targett and Lumsden, 1975) by transfer

of these tissues to normal recipients and subsequent examination of peripheral blood. Borrelia recurrentis, the causative organism of relapsing fever in man, undergoes a similar syndrome of waves of parasitaemia as is seen in trypanosome infection, and during the afebrile period between successive waves, infection cannot be detected in nor transmitted by blood.

The ability to evade host response and the action of drugs, and also to adopt an insusceptible form, is well illustrated in tuberculosis infections. The typical tubercle lesion, which is present in lung parenchyma and mediastinal lymph node, retains viable bacilli which even after many years can resume multiplication. Such fibrous or calcified lesions protect the organism from host defences and also promote a metabolically inactive state which prevents the action of bactericidal drugs.

The previous section of this chapter described a type of relapsing infection in mice after chemotherapy which can be ascribed neither to drug-underdosage nor drug-resistance. Rather, the results indicated that a situation similar to that described above may be operating, namely that the parasite may be present in a site inaccessible to the action of the drug or in a stage of development against which normally effective drugs are unable to act. The latter would suggest a stage in the life-cycle of the parasite akin to the amastigate form of T. brucei suggested by Ormerod and Venkatesan (1970, 1971a,b), or the "ocult" form (Goodwin and Rollo, 1955) which may be relatively unsusceptible to the action of drugs.

This section records the outcome of a series of experiments to identify the location of the cryptic infection during the aparasitaemic period following chemotherapy and to determine the morphology of the parasite during this phase.

MATERIALS AND METHODS

The mice and trypanosomes used in these experiments have been described in section one of this chapter.

Chemotherapy: This was done with Berenil (diminazene aceturate, Hoechst, Germany). The dose was 40 mg active principle/kg body weight, and was administered to mice as outlined on page

Preparation of tissue homogenates: Donor mice were exsanguinated by cardiac puncture under deep trichloroethylene anaesthesia (Trilene, I.C.I., England), and immediately dissected. Initially, all tissues were mechanically disrupted with a homogeniser (Silverson, London, England), but in later experiments, with the exception of brain, all tissues were teased apart through a fine wire sieve, using a rubber-tipped spatula, into sterile PSG. Whole mouse brains were transferred from the cranial cavity using forceps into a 2 ml syringe containing a small quantity of sterile PSG, and forced sequentially through hypodermic needles of progressively narrower gauge. After passage through 16 gauge, 19 gauge and 21 gauge needles, the tissue was of a consistency suitable for injection. Each cell suspension, derived from an entire organ, was then injected i.p. into 1 recipient, except in the case of liver whose size required that it be divided between 2 recipients. Four lymph nodes per animal (right and left popliteal and prefemoral) were pooled, disrupted and injected together into 1 recipient mouse. The volume of blood injected was 0.5 ml.

Ion-exchange chromatography: This was done by the miniature anion-exchange/centrifugation (AEC) technique of Lumsden, Kimber and Strange (1977). Briefly, 70 µl of blood from a capillary tube was introduced on to the surface of a DEAE-cellulose gel (DE52, Whatman, England) which had been prepared in a 2 ml syringe barrel. The blood sample was allowed to penetrate the surface of the cellulose bed, the buffer reservoir attached (PSG, pH 8.0) and 2 ml eluate collected in a pasteur pipette with a drawn-out, sealed end. This was centrifuged at 3000 x g for 15 minutes, and examined microscopically at the tip for trypanosomes.

Experimental design: The results from the first section of this chapter indicated that treatment with Berenil at 40 mg/kg resulted in an aparasitaemic period of at least 3 weeks duration following which recrudescence of parasitaemia occurred. Accordingly, it was on the initial phase following treatment that most attention was paid.

Mice were inoculated with 1×10^4 parasites i.p. and tail blood was examined after 5 days to confirm that they were infected. Treatment was given on day 21, and the mice were monitored over the ensuing 3 days to confirm the diminution of the bloodstream parasitaemia. On either the 7th or 14th day after treatment, the mice were killed, the organs removed, and homogenates of these organs injected into clean recipients. The absence of trypanosomes in the blood on the day of tissue transfer was confirmed by microscopic examination and the miniature AEC method. Tail blood

from the mice which received inoculation of tissues was checked every 2 days for the presence of parasites for the first 10 days after tissue transfer, and thereafter twice per week. Mice were judged to be uninfected only after 60 days had elapsed since tissue transfer without trypanosomes being detected in the blood-stream. Mice with positive wet films were immediately killed to avoid possible cross-infection within a cage. Between 3 and 6 donor mice in each experiment were not sacrificed after Berenil treatment and served as relapse controls.

RESULTS

The focus of the cryptic infection. The experiments from section 1 of this chapter established that a high incidence of relapse of infection occurred when a T. brucei infection in mice was treated after 3 weeks with Berenil, whereas no relapses occurred after chemotherapy of a 3 day infection. The absence of viable trypanosomes in the host after treatment at this early stage was confirmed by a tissue transfer experiment, the results of which are shown in Table 1.5. None of the tissues which were removed, 2 weeks after chemotherapy, from mice treated on the third day of a T. brucei TREU 667/1 infection were able to transmit infection to recipient animals.

However, when this experiment was done in mice whose infection was of 3 weeks' duration at the time of chemotherapy, infection was successfully transmitted from a proportion of mice (Table 1.6). The brains only from 3 of 8 mice established infection in recipient mice. Infection in these donors resided in no other location, since blood and other tissues failed to transmit infection. Mice from the donor group which were not killed developed parasitaemias 28-47 days after chemotherapy.

In both of these experiments, tissue disruption was done mechanically using a Silverson homogeniser, which produced easily injectable tissue homogenates. However it was possible that such treatment of tissues may also have disrupted trypanosomes, particu-

Table 1.5 Transmission of infection with tissue from mice 14 days after chemotherapy of a 3-day infection with T. brucei TREU 667/1.

No. of donors	Tissue				
	Blood	Brain	Liver	Kidney	Spleen
8	* 0/8	0/8	0/16	0/16	0/8

*No. of recipients parasitaemic/No. of recipients injected.

Six donor mice which were not sacrificed after Berenil treatment did not relapse after treatment.

Table 1.6 Transmission of infection with tissue from mice 14 days after chemotherapy of a 3-week infection with *T. brucei* TREU 667

Donor	Tissue				
	Brain	Blood	Liver	Kidney	Spleen
1	0/1*	0/1	0/1	0/1	0/1
2	0/1	0/1	0/2	0/1	0/1
3	0/1	0/1	0/2	0/1	0/1
4	0/1	0/1	0/2	0/1	0/1
5	0/1	0/1	0/2	0/1	0/1
6	1/1 (24)	0/1	0/2	0/1	0/1
7	1/1 (26)	0/1	0/1	0/1	0/1
8	1/1 (22)	0/1	0/2	0/1	0/1

( ) : day of patent parasitaemia after tissue transfer.

* No. of recipients parasitaemic/No. of recipients injected.

Six donor mice which were not sacrificed after Berenil treatment relapsed on days 28, 30, 30, 40, 40, 47.

larly since there is no information on the fragility of tissue forms compared to trypomastigates. For this reason, these two experiments were repeated, including heart, lung and lymph node tissues, and a more gentle tissue disruption method employed.

The result of the experiment involving tissue transfer from mice 2 weeks after day 3 treatment with Berenil was unaltered. Table 1.7 shows the outcome of transfer after a 3 week infection. Infection from 3 of 5 donors was successfully transmitted, again only from brain tissue. In those recipient mice which became parasitaemic, the prepatent periods were significantly shorter, 10, 12 and 14 days compared with 22, 24 and 26 days than in the previous experiments. However, whether the higher incidence of detection of infection in brain tissue and the apparently greater number of parasites present, as reflected by the prepatent periods in the recipient mice, is a direct consequence of more gentle handling of the material or merely between-experiment variation is unclear.

A higher incidence of transmission from brain was achieved from mice which had been infected with T. brucei 667/1 for 7 weeks before treatment (Table 1.8): brain homogenate from 6 of 8 mice produced parasitaemias between 7 and 27 days after inoculation into recipient mice. Spleen, liver, heart, lymph node, kidney and lung tissue failed to transmit infection. The absence of circulating trypanosomes, indicated by blood films and miniature AEC on the day of transfer was substantiated, as in the previous experiments, by the non-infectivity of donor blood.

Table 1.7 Transmission of infection with tissue from mice 14 days after chemotherapy of a 3-week infection with *T. brucei* TREU 667

Donor	Tissue						
	Brain	Blood	Spleen	Lung	Heart	Kidney	Lymph node
1	0/2	0/2	0/2	0/1	0/1	0/1	0/1
2	1/2 (14)	0/1	0/2	0/1	0/2	0/1	0/1
3	0/2	0/2	0/2	0/1	0/1	0/1	0/1
4	1/2 (12)	0/1	0/2	0/1	0/1	0/2	0/1
5	1/2 (10)	0/2	0/2	0/2	0/1	0/1	0/1

( ) : Day of patent parasitaemia after tissue transfer

No. of recipients parasitaemic/No. of recipients injected

Three donor mice which were not sacrificed after Berenil treatment relapsed on days 59, 59, 68.

Table 1.8 Transmission of infection with tissue from mice 7 days after chemotherapy of a 7-week infection with *T. brucei* TREU 667

Donor	Tissue							
	Brain	Blood	Spleen	Liver	Heart	Kidney	Lung	Lymph node
1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
2	1/1 (7)	0/1	0/1	0/1	0/1	0/1	0/1	0/1
3	1/1 (27)	0/1	0/1	0/1	0/1	0/1	0/1	0/1
4	1/2 (14)	0/2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	2/2 (6,6)	0/2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	2/2 (14,14)	0/2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	2/2 (14,21)	0/2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	0/2	0/2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

( ) : day of patent parasitaemia after tissue transfer

n.d. : not done

No. of recipients parasitaemic/No. of recipients injected

Previous experiments in section 1 had revealed that the phenomenon of relapse after chemotherapy was a feature common to several stabilates of T. brucei. Two other stabilates, T. brucei LUMP 1001 and T. brucei LUMP 571 were therefore examined to find if infection also resided in the brain tissue during the aparasitaemic period after drug treatment. Since the previous experiments involving these two stabilates, in particular LUMP 1001, indicated that relapses occurred in some cases as early as 3 weeks after chemotherapy, tissues were transferred on day 7 following Berenil treatment rather than day 14. This was done to eliminate the possibility of trypanosomes already being in the bloodstream at the time of tissue transfer.

With T. brucei LUMP 1001 blood transfer was negative, thus confirming the absence of circulating trypanosomes as indicated by wet film and miniature AEC examinations. All 24 recipient mice which received brain homogenate from the 12 donors developed parasitaemias (Table 1.9). All recipients were parasitaemic by day 24 except for one which became patent after 43 days. That the donors themselves would ultimately have developed a relapsing parasitaemia was indicated by the recrudescence of infection in the controls 24 to 30 days after Berenil treatment.

A similar pattern was observed with T. brucei LUMP 571. The brains of 6 donor mice transmitted infection to recipients, while the blood from the same donors was non-infective (Table 1.10). Relapse in control mice occurred between 40 and 54 days following chemotherapy.

Table 1.9 Transmission of infection with tissue from mice 7 days after chemotherapy of a 3-week infection with *T. brucei* LUMP 1001

No. of donors	Tissue	
	Blood	Brain
12	0/24	24/24*

*23/24 recipients were parasitaemic by day 24 after tissue transfer. Last mouse became patent on day 43.

No. of mice parasitaemic/No. of recipients injected.

Four donor mice which were not sacrificed after Berenil treatment relapsed on days 24, 25, 27, 30.

Table 1.10 Transmission of infection with tissue from mice 7 days after chemotherapy of a 3-week infection with *T. brucei* LUMP 571

No. of donors	Tissue	
	Blood	Brain
6	0/12	12/12*

*All mice became patients by day 17 after tissue transfer.

No. of mice parasitaemic/No. of recipients injected.

Three donor mice which were not sacrificed after Berenil treatment relapsed on days 40, 48, 54.

The indications from these experiments was that it was only in brain tissue that infective trypanosomes could be found after chemotherapy. The stage of development of the parasite, or the particular location of the parasite within the brain, and the number present could not be ascertained from the above information.

The number of trypanosomes in the brain after chemotherapy

An approximation of the numbers of trypanosomes present in the brain could be made in the above experiments by considering the prepatent periods in the recipients of brain tissue, but in order to obtain a more accurate assessment of trypanosome numbers, an infectivity titration of brain tissue was done. Seven days after Berenil treatment of a 21 day infection with T. brucei LUMP 1001, the brains were removed from 6 mice, pooled and homogenised. Ten-fold dilutions were made of the brain tissue in PSG, and groups of mice (6 per group) were inoculated with the dilutions of tissue. The volume of tissue which was injected into the 6 mice comprising the group receiving undiluted tissue homogenate was one-sixth of the total material, ie. the group as a whole received an equal share of "1 brain equivalent". Similar volumes of material were accordingly inoculated at the other dilutions. The results (Table 1.11) indicated that at this stage after chemotherapy of T. brucei LUMP 1001, the brain contained approximately 25,000 infective trypanosomes.

Morphology of trypanosomes in the brain

Brain homogenate was also examined for trypanosomes by

Table 1.11 Infectivity titration of brain tissue seven days after Berenil treatment of a twenty-one day infection with T. brucei LUMP 1001

Dilution of brain tissue	No. of recipients positive/Total
Neat	6/6
$1:10^1$	6/6
$1:10^2$	6/6
$1:10^3$	6/6
$1:10^4$	6/6
$1:10^5$	0/6
$1:10^6$	0/6

This corresponds to approximately 25,000 infective trypanosomes/brain, from the method of Lumsden, Herbert & McNeillage (1973)

applying a slurry of homogenate to the surface of a DE52 column (Lanham and Godfrey, 1970) and examining the eluate after centrifugation for trypanosomes. Examination of a wet preparation of the brain homogenate by light microscopy failed to detect any trypanosomes, nor were parasites seen on a Giemsa-stained smear of this material. However, after chromatography and centrifugation parasites were detected. Morphologically, these were trypomastigotes, and no amastigote forms were seen.

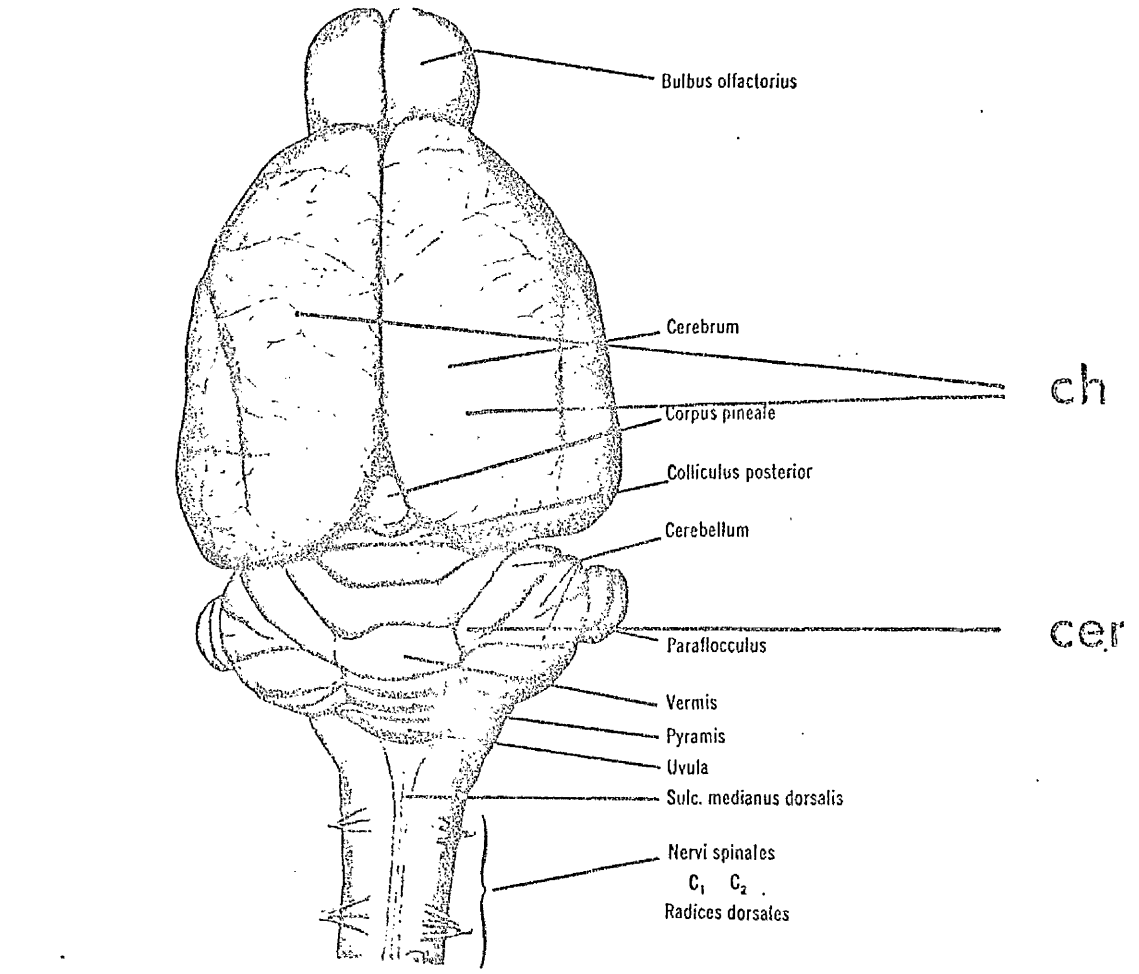
Location of trypanosomes in the brain

Using T. brucei TREU 667/1 and T. brucei LUMP 1001, an experiment was done to investigate the location of trypanosomes within the brain. Seven days after chemotherapy of a 3 week infection with each of these parasites, the brains were removed and dissected (Fig 1.10). Both cerebral hemispheres and the cerebellum were removed, the choroid plexuses were excised from both lateral ventricles, and the remainder of the brain was sectioned transversely to yield fore-brain, mid-brain, hind-brain and the upper section of spinal cord. These were then injected separately into individual mice, which were then monitored for infection. The results (Table 1.12) indicated that in the case of T. brucei LUMP 1001, the ability to transmit infection was possessed by each portion of the brain, while with T. brucei TREU 667 there appeared to be a localisation of trypanosomes in the fore- and mid-brain, while the other portions were non-infective to mice.

Fig 1.10

After removal of the brain from the cranial cavity, the mouse brain was dissected as shown opposite. After removal of the cerebral hemispheres (ch) and the cerebellum (cer), the choroid plexuses were removed from the exposed lateral ventricles (TOP). The remainder of the brain was dissected transversely with 3 parallel incisions to yield forebrain (F), midbrain (M), hindbrain (H) and spinal cord (SC). (BOTTOM).

Figure compiled from Sissons (1975).


Bulbus olfactorius
Cerebrum
ch
Corpus pineale
Colliculus posterior
Cerebellum
cer
Parafloccolus
Vermis
Pyramis
Uvula
Sulc. medianus dorsalis
Nervi spinales
C_1 C_2 .
Radices dorsales

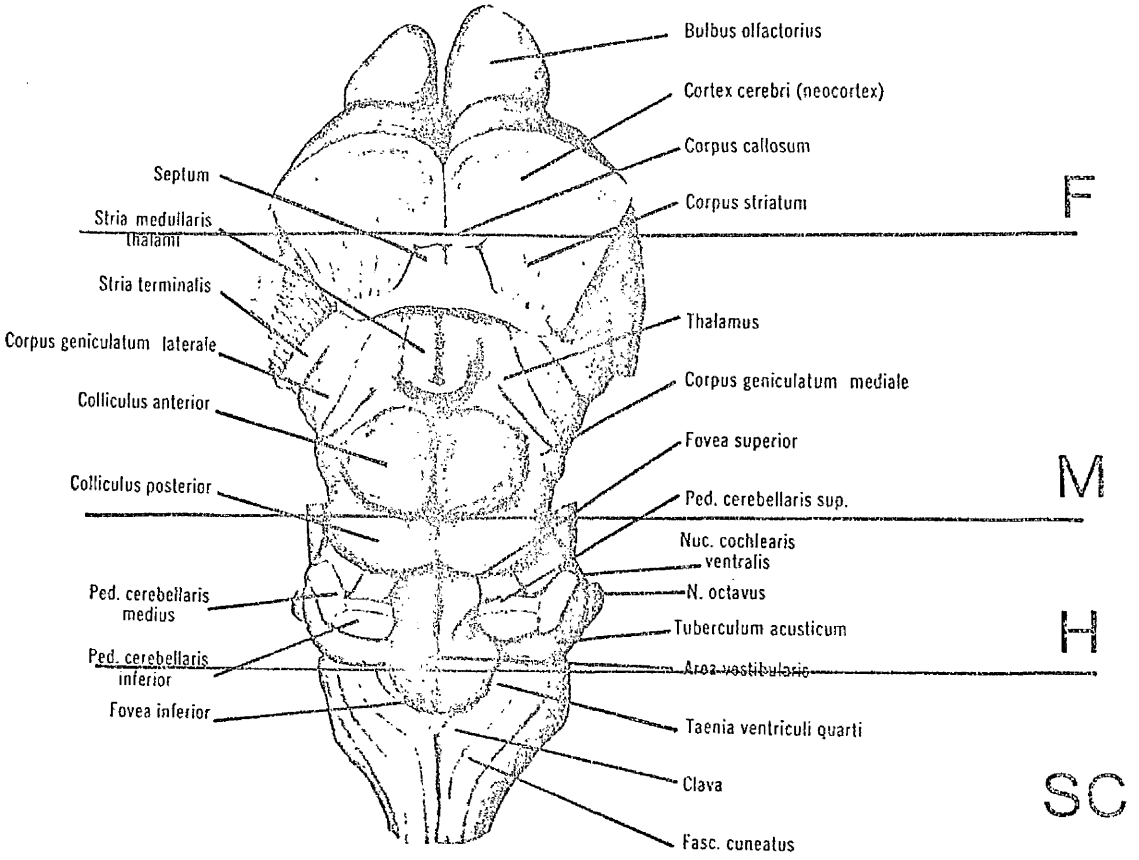

Bulbus olfactorius
Cortex cerebri (neocortex)
Corpus callosum
Septum
Corpus striatum
F
Stria medullaris thalami
Stria terminalis
Thalamus
Corpus geniculatum laterale
Corpus geniculatum mediale
Colliculus anterior
Fovea superior
M
Colliculus posterior
Ped. cerebellaris sup.
Nuc. cochlearis ventralis
N. octavus
Ped. cerebellaris medius
Tuberculum acusticum
H
Ped. cerebellaris inferior
Area vestibularis
Fovea inferior
Taenia ventriculi quarti
Clava
SC
Fasc. cuneatus

Table 1.12 Infectivity of different sections of mouse brain 7 days after chemotherapy of a 3-week infection with either *T. brucei* TREU 667/1 or *T. brucei* LUMP 1001

	T. brucei	
	LUMP 1001	TREU 667/1
Forebrain	+	+
Midbrain	+	+
Hindbrain	+	-
Cerebral Hemispheres	+	+
Choroid Plexus	+	-
Cerebellum	+	-
Spinal Cord	+	-

The focus of infection after chemotherapy of T. congolense infection

A tissue transfer experiment similar to those described above was done with a T. congolense population which had previously shown a high incidence of relapse after chemotherapy of a 3-week infection. This population had been stabilated from a mouse which relapsed 35 days after a 3 week treatment with 40 mg/kg Berenil (Fig 1.9c). Brain, spleen, liver, lung, kidney, heart, lymph node and blood were transferred to recipient mice on the 7th day after chemotherapy of a 3 week infection with this stabilate in C57Bl mice. This experiment was done on 3 separate occasions and the distribution of infective tissue was different in each case, with no obvious pattern being seen. However, taking the 3 experiments together, infective parasites were transferred after chemotherapy from each tissue type.

DISCUSSION

A significant problem in the chemotherapy of animal trypanosomiasis is the occurrence of relapses after treatment. When a trypanocidal drug fails to clear the circulating parasitaemia or if parasites reappear in the bloodstream after a very short aparasitaemic phase (8-10 days) the likely reason is either underdosage of the drug or drug resistance of the parasite. In contrast, a prolonged aparasitaemic phase after chemotherapy followed by the eventual reappearance of the parasite has usually been attributed to reinfection.

The results from the first section of this chapter provided the basis for the experiments described in section 2, namely that after a 21 day infection in mice, T. brucei assumes a form of development or a location within the host in which it is insusceptible to chemotherapy and is thus capable of re-establishing a bloodstream infection.

A series of tissue transfer experiments with several different stocks of T. brucei provided consistent evidence that during the aparasitaemic phase following chemotherapy with Berenil, viable trypanosomes are present only in the brain. Thus, tissue forms (Soltys et al, 1969; Soltys and Woo, 1969, 1970) which are presumably present in spleen and liver and other tissues at the time of chemotherapy are as accessible and susceptible to the drug as the trypomastigotes in the bloodstream. Consequently, these tissues are unable to establish infection when injected into

susceptible mice. With brain tissue, however, successful transmission is possible, which suggests 2 possible explanations. First, the environment in the brain promotes the formation of a drug-insusceptible form of the parasite, which in the absence of trypanocidal levels of drug reverts to a replicative form which re-emerges and initiates a bloodstream infection. Alternatively, and more probable, is that there exists a barrier through which the trypanosome may penetrate but the commonly-used trypanocidal drugs may not. Once beyond this barrier, the parasite may assume an amastigote-like form (Ormerod and Venkatesan, 1970, 1971a,b) or remain as a trypomastigote, either in the neuropil or in the cerebrospinal fluid (CSF).

Such a barrier exists in mammals, the blood brain barrier, and it is operative at 3 levels: first, at the endothelial lining of blood vessels within the brain and in the pia-mater, secondly, at the epithelial lining of the choroid plexuses, and thirdly, at the mesothelium which forms the outer lining of the sub-arachnoid spaces. At all 3 levels, complete tight junctions or zonula occludens are found between the cells, preventing leakage of large molecules (Brightman and Reese, 1969). In addition, the endothelial cells of blood vessels within the brain have a limited capacity to endocytose and do not contain contractile elements, thus rendering them insensitive to inflammatory mediators such as histamine, serotonin and noradrenalin. The level at which the trypanosomes cross the blood-brain barrier and whether their passage requires damage or increased permeability of the barrier

is unknown. In acute infections with T. brucei in dogs and mice (Poltera et al, 1980), large numbers of trypanosomes are found extravascularly in the choroid plexuses prior to invasion of the brain. In this site they are still outside the blood brain barrier, and presumably accessible to drugs. However, the early presence of organisms in the choroid plexus suggests that this may be the site where they initially cross the blood-brain barrier into the CSF. Once within the CSF in the ventricles, they can pass directly into the sub-arachnoid space and from there into the Virchow-Robin spaces: as there is no functional barrier either at the level of the ependymal lining of the ventricles or at the pia-olial lining of the Virchow-Robin spaces (Rapoport, 1976), it is likely that trypanosomes in the CSF will have ready access to the neuropil.

Two experiments were done to obtain information on the location and numbers of parasites beyond the blood-brain barrier. An infectivity titration of brain tissue after chemotherapy indicated that T. brucei LUMP 1001 was present in very large numbers after chemotherapy, and from the dissection studies it appeared that the parasites were widely disseminated through the tissue. T. brucei TREU 667/1 was less widely dispersed, but this may be a result of the timing of the experiment.

It is technically very difficult to obtain uncontaminated samples of CSF from mice. Central nervous system (CNS) involvement in human patients is confirmed by examination of CSF after

lumbar puncture, and trypanosomes can often be detected microscopically in the fluid. In view of the wide dissemination of trypanosomes in the brain of mice after chemotherapy and the fact that CSF was no doubt present in each brain tissue preparation, it is possible that parasites were present throughout the CSF. However, in the absence of a sample of CSF this possibility remains unconfirmed.

Also unconfirmed is the morphology of the parasite within the CNS. Chromatographic studies revealed only the presence of trypomastigotes, but the existence of other forms, which may for example, possess a different surface charge, and thus be retarded in a DE52 column, cannot be eliminated. This is particularly relevant if one considers the prepatent periods of the mice which received brain tissue. In some cases, the prepatent period was 6 days, in others 6 weeks elapsed before circulating parasites were detectable. Such a difference may be due to the status of the parasite in the brain, a rapid establishment of parasitaemia in the recipient arising from trypomastigotes, whereas a delayed infection may be due to amastigote-like forms which require time to revert to blood forms. However, stained smears from brain tissue did not reveal the presence of morphological types resembling those described previously (Ormerod and Venkatesan, 1971b; Mattern and Felici, 1972).

Furthermore, a result from section one of this chapter may be interpreted as evidence of a drug insensitive form of the parasite existing within the CNS. Most of the commonly used

trypanocidal drugs do not efficiently cross the blood-brain barrier (Gutteridge and Coombs, 1977), but the ionic character of Melarsen enables it to pass from the bloodstream to the CSF and for this reason it has been used in the treatment of human cases of the Gambian disease (Butler, Duggan and Hutchinson, 1957). In view of the inability of Melarsen to produce permanent cure in mice (Table 1.3), the possibility of there existing a less susceptible form of the parasite beyond the blood-brain barrier, while perhaps unlikely, cannot be eliminated.

The results of the experiments with T. congolense are less easy to interpret, and it is likely that the mechanism of relapse is different than that described above for T. brucei. T. congolense is considered to be strictly a plasma parasite confined to the circulatory system (van de Ingh et al, 1976; Losos et al, 1973), but it has recently been demonstrated that T. congolense can multiply and persist in connective tissues (Luckins and Gray, 1978). If the parasite has the capability of populating the tissues of a host during an infection, its persistence in such a location may influence the efficiency of chemotherapy, particularly since as discussed earlier, it has been suggested that such forms may be relatively unsusceptible to the action of drugs (Goodwin and Rollo, 1955). However, there is no evidence for there being specific localisation within the CNS of the host as appears to be occurring with T. brucei.

Recent studies have provided information on the lesions

resulting from CNS involvement with T. brucei species parasites in mice, and are in accord in concluding that T.b.gambiense (Van Marck et al, 1981a,b), T.b.rhodesiense (Fink and Schmidt, 1979) and T.b.brucei (Poltera et al, 1980) produce a meningo-encephalitis, with inflammation and parasitism of the choroid plexus. In all reports, histological or electron microscopic evidence eliminated the occurrence of amastigote forms of the parasite.

The sequential involvement of brain structures described by Poltera et al (1980), provides an explanation for both the invasion of the CNS in chronic trypanosome infections and the consequent efficacy of chemotherapy. Trypomastigotes may first migrate from the vascular compartment into the interstitium of the choroid plexus, possibly favoured by an increase in vascular permeability involving circulating immune complexes and complement activation. At this stage they are accessible to chemotherapeutic drugs. Trypanosomes localised in the tissues may then trigger a local immunologically mediated inflammatory reaction favouring their migration into the CSF and further invasion of other cerebral structures where they are inaccessible to drug action.

CHAPTER II

MATERNALLY-DERIVED IMMUNITY TO TRYPANOSOMIASIS

INTRODUCTION

It has long been recognised that diseases such as mumps, measles, diphtheria, scarlet fever and some other infections of man and of animals rarely affect the very young. This is the result of inheritance from the mother of resistance to a specific infective agent to which she has previously been exposed or against which she has been vaccinated. Thus the mother has an active immunity against the agent which is passively transmitted to her offspring, a sequence of events which has been known for many years. Young mammals are unable to produce antibodies themselves for some time after birth, and they acquire these antibodies, which are associated with their immunity, from the mother, either whilst in the uterus before birth, or through the colostrum after birth. The relative importance of the route of transfer varies from species to species (Table 2.1).

The concept of the transmission of immunity from a mother to her offspring is by no means a new one. Ehrlich (1892) administered the toxalbumins recin, robin and abrin to pregnant mice which subsequently became immune to them. When the young were born, they were also immune, and this immunity was considered to be passive, the specific antibodies being transferred to the foetus in utero across the placenta and also through the milk immediately after birth. When the young from normal mothers were allowed to suckle from immune mice, they too became immune, showing that immunity could be transferred via the milk. When,

Table 2.1 Classification of placentation according to the intervening tissues and the correlation with time of transfer of immunity from mother to offspring

Type of Placentation	Animal Species	Endothelium	Connective tissue	Epithelium	Trophoblast	Mesenchyme	Endothelium	Time of Antibody Transmission Before birth	After birth
Epithelio-chorial	Horse Pig	+	+	+	+	+	+	-	+
Syndesmo-chorial	Ox Sheep Goat etc	+	+	-	+	+	+	-	+
Endothelio chorial	Dog Cat	+	-	-	+	+	+	±	+
Haemochorial	Man	-	-	-	+	+	+	+	-
Haemoendothelial*	Rat Mouse Rabbit Guinea pig	-	-	-	-	-	+	±	±

*Although the rat and mouse have haemoendothelial placentation, it is stated by some authors (e.g. McGirr, 1947) that transmission occurs mainly after birth.

however, the young of immune mice were allowed to suckle with non-immune mice, they displayed some immunity, though to a much less degree than in the previous group. He concluded that while immunity appeared to be obtained by the young to a very small extent whilst still within the uterus, most was being transmitted through the milk.

The importance of colostrum to the new-born calf was investigated almost sixty years ago by Smith and Little (1922a) who described its protective effect against white scours in a small group of experimental animals. Of twelve newborn calves deprived of colostrum, eight died and a ninth was destroyed *in extremis*. All of the ten calves that were allowed to receive colostrum survived, and the authors commented that "the calf deprived of colostrum lacks something which permits intestinal bacteria to invade the body and multiply in the various organs". Whatever this something may have been, they were able to show that cow's serum might under some circumstances be a more or less successful replacement for it (Smith and Little, 1922b).

In many mammals, the cornerstone of early postnatal survival of infection is the acquisition of passive immunity from the mother through suckling. Such passive immunity in rats or mice has been demonstrated to many infectious agents or toxins: *Plasmodium berghei* (Bruce-Chwatt and Gibson, 1956; Terry, 1956); *Rickettsia* (Jo, 1953); *Toxoplasma* (Lewis and Markell, 1958); *Cysticercus fasciolaris* (Miller, 1935); *Salmonella* (Halliday, 1955); *Brucella abortus* (Halliday and Kekwick, 1960); *Trichinella spiralis*

(Mauss, 1940); tetanus toxin (Kosunen and Halonen, 1963); and many viruses eg blue tongue virus (Svehag and Gorman, 1963).

Although the development of immunity to trypanosomiasis in domestic animals is only occasionally a sequel to natural infection, protective immunity against a particular antigenic variant can be readily induced by a variety of vaccination procedures (reviewed by Terry, 1976; Clarkson, 1976; Murray and Urquhart, 1977; Holmes, 1980). Such immunity depends largely on serum antibody, and protection can be achieved by passive transfer of homologous antibody, as has been shown with T. gambiense (Seed and Gam, 1966a,b; Takayanagi, Kambara and Enriquez, 1973), T. brucei (Watkins, 1964), and T. rhodesiense (Seed, 1963). While both specific IgG and IgM are protective (Seed, 1972; Takayanagi et al, 1973; Zahalsky and Weinberg, 1976), Takayanagi et al (1973) found that IgM was more effective at neutralising T. gambiense, smaller quantities being required to neutralise a given number of trypanosomes, whereas Zahalsky and Weinberg (1976) suggested that IgG was more effective in producing protection to T. brucei in mice.

The experiments which form the basis of this chapter were stimulated by studies reported over 40 years ago. Following the failure by Minning (1936) to demonstrate the transmission of immunity to Trypanosoma lewisi from mother rats to their offspring, and a similar failure by Collier (1931) with mice infected with T. cruzi, Culbertson (1938) showed that the young of mother rats recently recovered from a T. lewisi infection were immune for the first few weeks of life to infection with this parasite. This

immunity was transmitted both by milk and colostrum, and partial immunity could be transmitted by specific antiserum *per os* (Culbertson, 1939). A similar result was observed in suckling mice whose mothers had recovered from a *T. duttoni* infection or been given passive injections of immune serum (Culbertson, 1940), and also in *T. cruzi* infections, where a normally fatal consequence to infection in young rats was modified to a mild abortive infection by suckling recovered or passively immunised mothers (Kolodny, 1939).

In the present study, the effectiveness of maternally-derived immunity to *T. brucei* and *T. congolense*, and the inter-relationship of passively-acquired immunity and trypanocidal therapy, have been investigated.

MATERIALS AND METHODS

Mice. These were CFLP and C57Bl/6 mice from Anglia Laboratory Animals (Cambridgeshire, England) and the Animal Breeding Research Organisation (ABRO, Edinburgh, Scotland) respectively. All pregnant mothers had been synchronously mated before despatch from the suppliers.

Trypanosomes. *T. brucei* TREU 667, and *T. congolense* GVR1 have have been described in Chapter 1 (page 36). The *T. brucei* stock produced infections in CFLP mice of over 6 weeks duration. Similarly, *T. congolense* GVR1 results in a chronic syndrome in C57Bl/6 mice (see Chapter 3).

Infections. Mothers were infected on the 14th day of pregnancy by intraperitoneal (i.p.) injection of 1×10^5 trypanosomes, prepared from frozen stabilates, in phosphate-buffered saline glucose (PSG), pH 8.0. Since the gestation period of mice is 19-21 days (Bronson, Dagg and Snell, 1975), this infection ensured that mothers were at or around peak parasitaemia at parturition, and unless otherwise stated, such mothers remained infected throughout the experiment. Offspring were challenged (i.p.) with 1×10^4 trypanosomes.

Chemotherapy. Diminazene aceturate (Berenil, Hoechst, Germany) was used at a dose of 40 mg active principle/kg body weight. In newborn mice (whose weight at birth is approximately 1.5 to 2.0 g), this was given on the day of birth as a single subcutaneous (s.c.)

injection in the scruff, using a micro-syringe (Hamilton Instruments, England) in a volume of 25 µl. Where required, pregnant mothers were treated i.p. with a single dose of Berenil the day before parturition.

Estimation of parasitaemia. Tail blood from the mice was examined for trypanosomes by the wet film technique. Mothers were monitored every 2 days until one week after parturition, and thereafter once per week. Similarly, challenged offspring were examined every 2 days until 2 weeks after infection, and subsequently twice per week. Such mice were judged to be immune if by 40 days after challenge they remained negative for trypanosomes.

Vertical transmission of infection. As part of the culling procedure of litters born of infected mothers, one or two newborn were killed and bled out at birth to screen for the possible presence of congenital infection: this was done by examination of blood by the wet film technique and mouse sub-inoculation. Newborn were judged to be negative for trypanosomes if, by 40 days after sub-inoculation, recipients of their blood remained aparasitaemic.

Fostering procedure. In each experiment, the groups were set up in duplicate. This was done to ensure that a mother was available as foster parent as soon as a litter was born, since there was no guarantee that despite synchronous mating, the mothers would reach full term on the same day. Thus, for each paired group, the

first litter born was destroyed to enable its mother to accept a foster litter. In order to optimise the chances of a mother accepting its foster litter, newborn were transferred to the cage of a mother, never vice-versa, and the young mice were handled with gloves dusted with the bedding of the recipient cage. The nest prepared by the mother for its own litter was retained. Thus it was hoped that by reducing stress to the mother and "camouflaging" the foster litter, she would be encouraged not to reject her new family. As a result of this procedure, the incidence of abandoned or devoured litters was kept below 25%.

Once the litter had been accepted by its foster parent, it was culled to comprise a maximum of 10 neonates (in some instances mothers gave birth to as many as 16 infants). Litters were weaned to 21 days of age.

In vitro neutralisation of trypanosomes with Berenil. Doubling dilutions of Berenil were done in distilled water from 256 µg active principle per ml to 2 µg per ml. To 0.6 ml of each dilution was added 0.6 ml of trypanosome suspension in 10% foetal calf serum in PSG, containing 2.5×10^6 organisms/ml. After incubation at 37°C for 2 hours, the contents of each tube were inoculated into a group of 6 mice. Thus each mouse received 2.5×10^5 trypanosomes which had been incubated in Berenil at a final concentration of between 1 and 128 µg/ml. These were then examined over the following thirty days for the presence of trypanosomes.

Trypanosome clearance assay. The development of this assay forms

the basis of chapter IV. Briefly, trypanosomes were harvested by ion-exchange chromatography (Lanham and Godfrey, 1970) from mice with fulminating parasitaemias which had been injected (i.v.) 24 hours previously with 5 mCi of the radioisotope ^{75}Selenomethionine. The parasite suspension was diluted such that a volume of 0.2 ml contained approximately 6,000 counts per minute (cpm). This corresponded to 5 x 10^7 trypanosomes. Test mice were injected i.v. with this labelled trypanosome suspension and sacrificed 60 min later. A terminal blood sample was collected, the liver and spleen excised and each was assayed for radioactivity in a gamma-counter (Packard tri-carb 3330, U.S.A.). The percentage distribution of radioactivity was then calculated.

RESULTS

Vertical transmission of infection. No offspring born of infected mothers were found to be infected themselves at birth. Over 100 neonates were screened by microscopic examination of blood or subinoculation of blood into susceptible recipients, both methods failing to reveal the presence of trypanosomes.

Transmission of immunity to young mice. To investigate if immunity to trypanosome infection was transmissible from a mother to newborn mice, and in order to determine if this immunity was transferred to offspring before or after birth, an experiment was done involving 6 series of mice. In Series I, mothers infected with T. brucei TREU 667/1 were allowed to keep their own litters. A group of uninfected mothers and their own litters comprised Series II. Series III consisted of a group of normal mothers on which were fostered the litters of infected mothers, and Series IV was the reciprocal of Series III, namely a group of infected mothers which received litters from non-infected mothers. Series V and VI resembled I and II, except foster litters were used. Thus Series V comprised infected mothers with a foster litter from an infected mother, and Series VI was made up of normal mothers and the offspring of normal mothers. Series II and VI also served as challenge controls for the experiment. In each Series, there were 3 groups of mice, which were challenged 16, 23 and 31 days after birth respectively.

Litters which were born of uninfected mothers, or were

fostered after birth to an uninfected mother regardless of whether or not the true parent was infected (Series II, III and VI), all became infected after challenge (Table 2.2). On the other hand, litters which suckled after birth an infected mother, irrespective of whether or not they themselves were born of an infected or normal parent, were totally immune when challenged 16 days after birth, approximately 55-60% were immune if challenged 23 days after birth, with the remainder exhibiting an extended prepatent period, whereas all mice were completely susceptible to challenge by 31 days of age.

A similar experiment was done in C57Bl/6 mice with T. congolense GVR1. From a pilot study (results not shown), the protection afforded young mice appeared to be of a shorter duration with this parasite compared to T. brucei TREU 667/1. Accordingly, the challenge dates were brought forward in this experiment. The results (Table 2.3) were similar to those described above for T. brucei. All mothers infected with T. congolense GVR 1 conferred immunity on their sucklings which waned to a non-protective level between 14 and 19 days after birth. This was the case irrespective of whether or not the young mice were the natural offspring of the parent. In contrast, suckling a normal mother resulted in complete susceptibility to challenge, even in mice which were the progeny of an infected mother.

Clearance of ^{75}Se-labelled Trypanosoma brucei TREU 667/1 in mice receiving antibody from the mother. The ability of mice receiving

Table 2.2 Transmission of immunity against T. brucei TREU 667/1 infection from mother to offspring

Day of challenge after birth	Number of offspring which fail to develop parasitaemia/Total in litter					
	Series I	Series II	Series III	Series IV	Series V	Series VI
16	7/7	0/10	0/9	8/8	8/8	0/7
23	5/8*	0/8	0/6	5/9*	4/7*	0/8
31	0/6	0/8	0/8	1/5	0/7	0/8

*In mice which became infected, the pre-patent period was 8-10 days, compared with 4 days in the controls

Table 2.3 Transmission of immunity against *T. congolense* GVR1 infection from mother to offspring

Day of challenge after birth	Number of offspring which fail to develop parasitaemia/Total in litter					
	I	II	III	IV	V	VI
10	8/9	0/8	0/8	7/7	6/6	0/6
14	8/10	0/7	0/9	7/7	7/8	0/9
19	0/5*	0/5	0/5	0/6*	0/6*	0/6

*The pre-patent period in mice in these groups was 7-10 days, compared to 5 days in controls.

colostrum/milk from an infected mother was measured by a clearance assay using homologous trypanosomes labelled with ^{75}Selenomethionine. This was done in mice aged 16, and 31 days of age which had been suckling with their own mother or a foster mother. The result is shown in Table 2.4, and correlates exactly with the results obtained with live challenge (Table 2.2). High liver uptake was achieved with mice of 16 days of age suckling an infected mother, but this activity had waned by 31 days of age. Hepatic clearance was absent in all mice suckling an uninfected mother.

Antigenic priming. From the first experiment, the duration of immunity to T. brucei TREU 667/1 conferred by suckling an infected mother, was in the region of 25 days. Termination of the capacity to transmit immunoglobulin from the intestine to the circulation occurs comparatively suddenly in the mouse between 14 and 16 days of age, there being no detectable transmission in a normal 17 day old mouse (Halliday, 1955, 1959). To determine if it is possible to prime an infant during the time it is passively acquiring its immunity from the mother, offspring were challenged on day 16, when intestinal transmission is ceasing but the young mouse is still protected, and subsequently rechallenged when maternally-derived immunity has normally waned. The results are shown in Table 2.5. As in the previous experiment, young mice challenged at 16 days of age were completely resistant to infection (Group A), whereas by 33 days of age they were completely susceptible (Group B). However, rechallenge of Group A on day 33 (17 days after the priming challenge) failed to establish infection, indicating that

Table 2.4 Clearance of ^{75}Se-labelled *T. brucei* TREU 667/1 in mice receiving colostrum/milk from the mother

Mice	Day	Spleen	Liver	Blood
Infected mother suckling own litter	16	4.6*	68.5	4.6
	31	4.6	10.8	65.2
Infected mother suckling foster litter	16	4.6	62.0	4.6
from uninfected mother	31	4.6	8.7	58.6
Uninfected mother suckling own litter	16	4.6	12.3	66.1
	31	4.6	13.1	73.2
Uninfected mother suckling foster litter	16	4.6	9.9	68.0
from infected mother	31	4.6	11.6	63.0

* % injected activity

Table 2.5 Antigenic priming

	Day of challenge		
	16	33	44
Group A[+]	9/9*	8/9	-
" B	-	0/8	-
" C	9/9	-	0/9
" D	-	-	0/8
Challenge controls	5/5	5/5	5/5

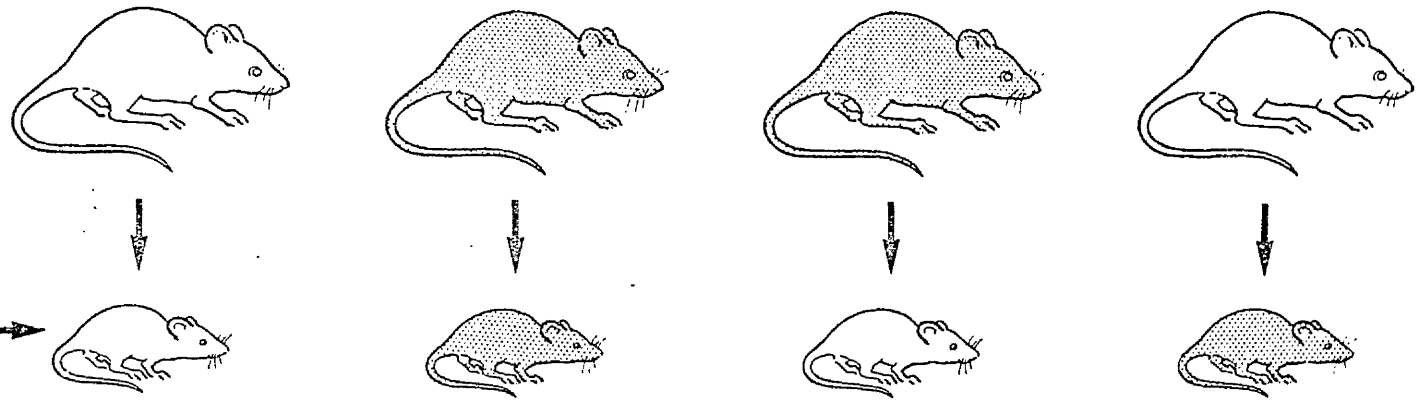
[+]All four groups were offspring of infected mothers, from which they suckled until weaning at 21 days of age.

*Number of offspring which fail to develop parasitaemia/total in litter.

Fig 2.1

Transmission of immunity in mice to T. brucei TREU 667/1 infection. This figure summarises the result of the first experiment, which showed that offspring were immune to a homologous T. brucei challenge for over 3 weeks after birth, providing that they suckled a mother infected with the parasite. This occured even if the young mice were themselves born of an uninfected parent. In contrast, a mouse which was suckled by an uninfected mother was completely susceptible to challenge, even if its natural parent was itself infected.

An identical situation occurred with T. congolense GVR1 infection, with a slightly different time scale.



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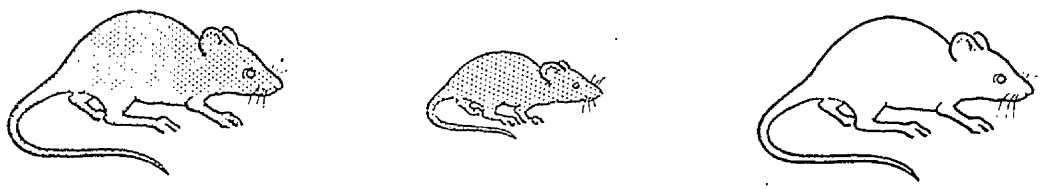


Normal mother

Offspring of normal mother

T. brucei Infected mother

Offspring of infected mother

priming of the host, despite the presence of passively acquired antibody, had occurred. The priming challenge had lost its effectiveness by 44 days of age (28 days after priming), showing that the active immunisation was no longer lasting than passively acquired immunity (Groups B and C) and was probably eliciting a primary response in the host.

Berenil prophylaxis in mice. The trypanocidal drug Berenil is used extensively in the treatment of trypanosomiasis in Africa. There are very few reports of drug resistance developing against it, which has been ascribed to its rapid rate of excretion which also accounts for its lack of prophylactic activity. However, pronounced prophylactic activity has been reported on several occasions (see p. 55 , Chapter 1), and consequently its effect on neonatal and adult mice was investigated in the present study. First, groups of neonatal mice which were the offspring of normal mothers were injected s.c. with 40 mg/kg Berenil on the day of birth, and challenged with 1×10^4 T. brucei TREU 667/1 either at 16, 23, 31, or 41 days of age. All infants were suckled normally by the mother until being weaned at 21 days of age. The results are shown in Fig 2.2. All 11 mice challenged on the 23rd day of age were protected, but by 31 days of age, despite there being a slight residual effect of the Berenil as reflected by an extended prepatent period in 6 out of 7 mice (6 days compared with 4 days), all mice became infected.

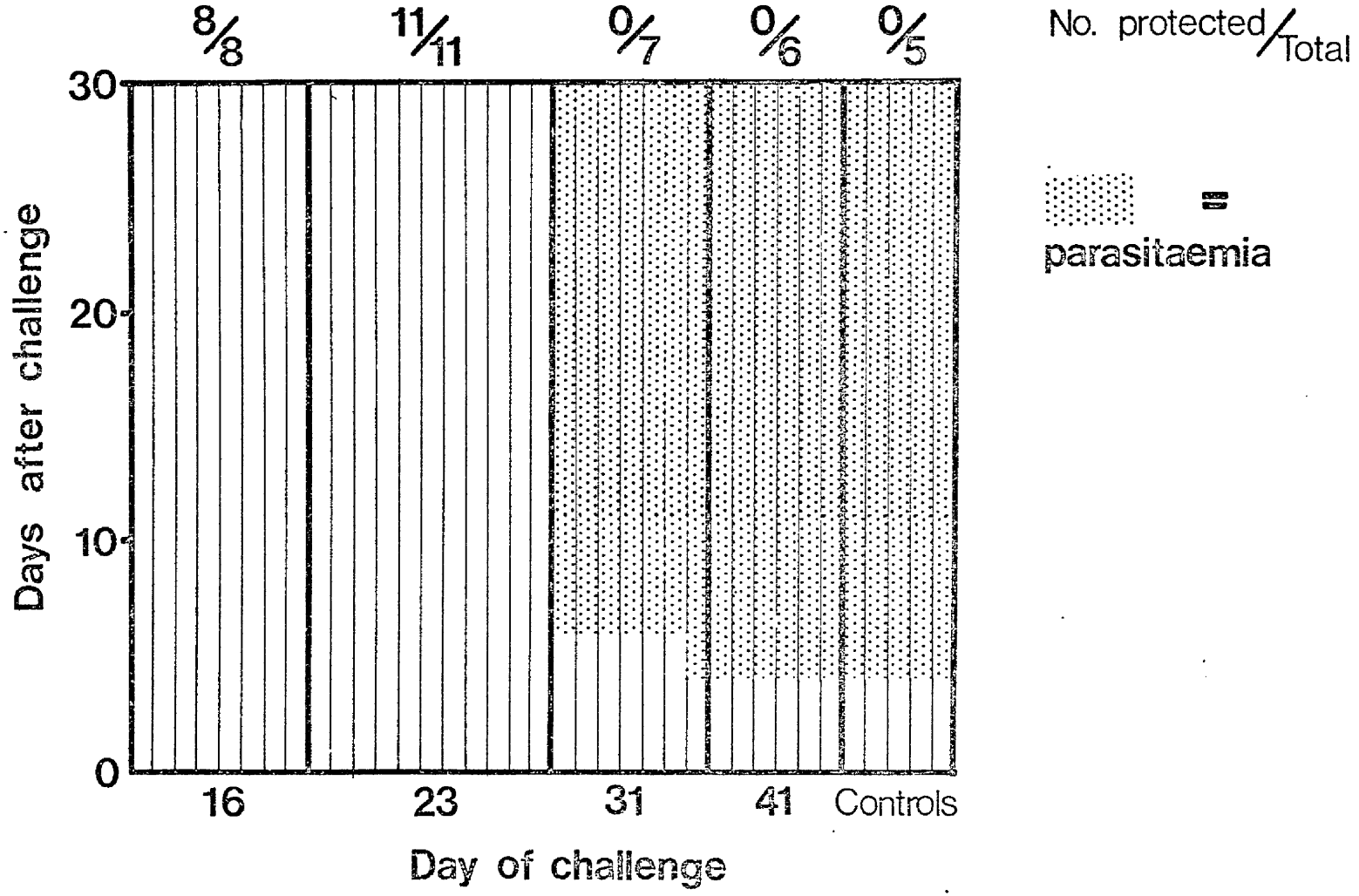
This experiment was also done in adult mice. Female

Fig 2.2

Berenil prophylaxis in neonatal mice.

The appearance of trypanosomes in individual mice challenged with T. brucei TREU 667/1 on different days after prophylactive treatment with 40 mg/kg Berenil at birth.

40mg/kg berenil prophylaxis in neonatal mice



CFLP mice aged between 6 and 8 weeks were injected s.c. with either 4 mg/kg or 40 mg/kg Berenil, and challenged with T. brucei TREU 667/1 either 3, 7, 14, or 21 days later (Fig 2.3). At the lower dose, trypanocidal levels of Berenil were present 7 days after treatment, 5 of 8 mice being completely protected against the challenge with the remaining 3 exhibiting a markedly prolonged prepatent period (14 days compared to 4 days in controls), but by 14 days the trypanocidal activity had virtually disappeared. As might be expected, 40 mg/kg Berenil afforded a greater degree of protection, with 2 of 5 mice completely eliminating the challenge and residual activity being evident in the remaining 3 mice.

In contrast, when adult C57Bl/6 mice were challenged with T. congolense GVR 1 after treatment with 40 mg/kg Berenil, very little prophylactic activity was evident (Fig 2.4). A small level of activity was seen in 2 of 8 mice challenged on day 7 after treatment as reflected by prepatent period, but even in mice challenged only 3 days after treatment, complete protection was not seen. All animals had prolonged prepatent periods (13 to 17 days compared to 4 days in controls) but in all cases the parasite became established. The possibility that this was the result of the presence of a drug resistant population of trypanosomes was discounted, since this same stock is permanently eliminated from mice if treated with Berenil 3 days after infection in mice (see Chapter 1).

Combined immune- and chemo-prophylaxis against T. brucei TREU 667/1.

From the above experiments, protection against trypanosome challenge

Fig 2.3

Berenil prophylaxis in adult mice.

The appearance of trypanosomes in individual adult mice challenged with T. brucei TREU 667/1 on different days after prophylactic treatment with either 4 mg/kg or 40 mg/kg Berenil.

Berenil prophylaxis in adult mice

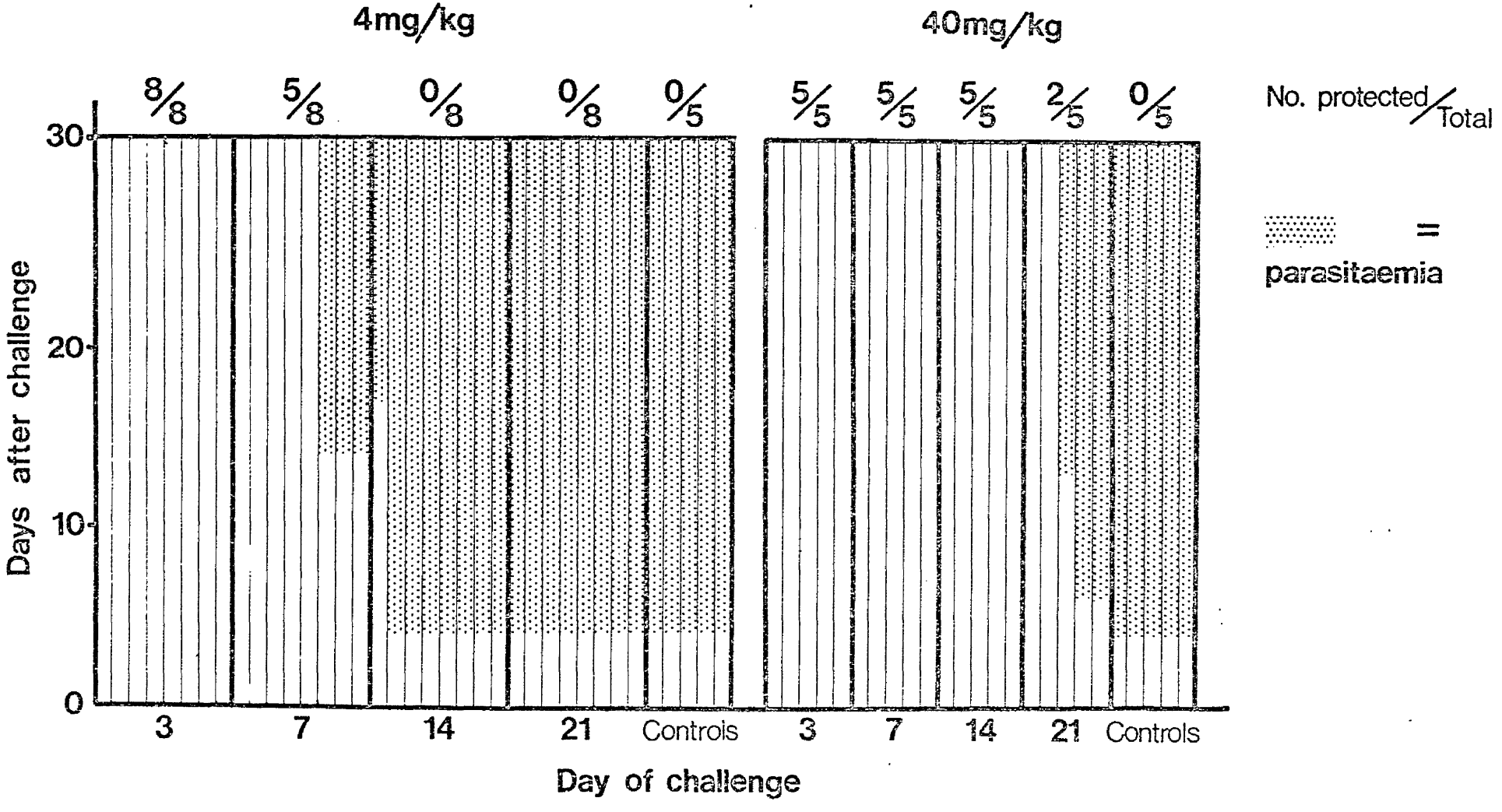
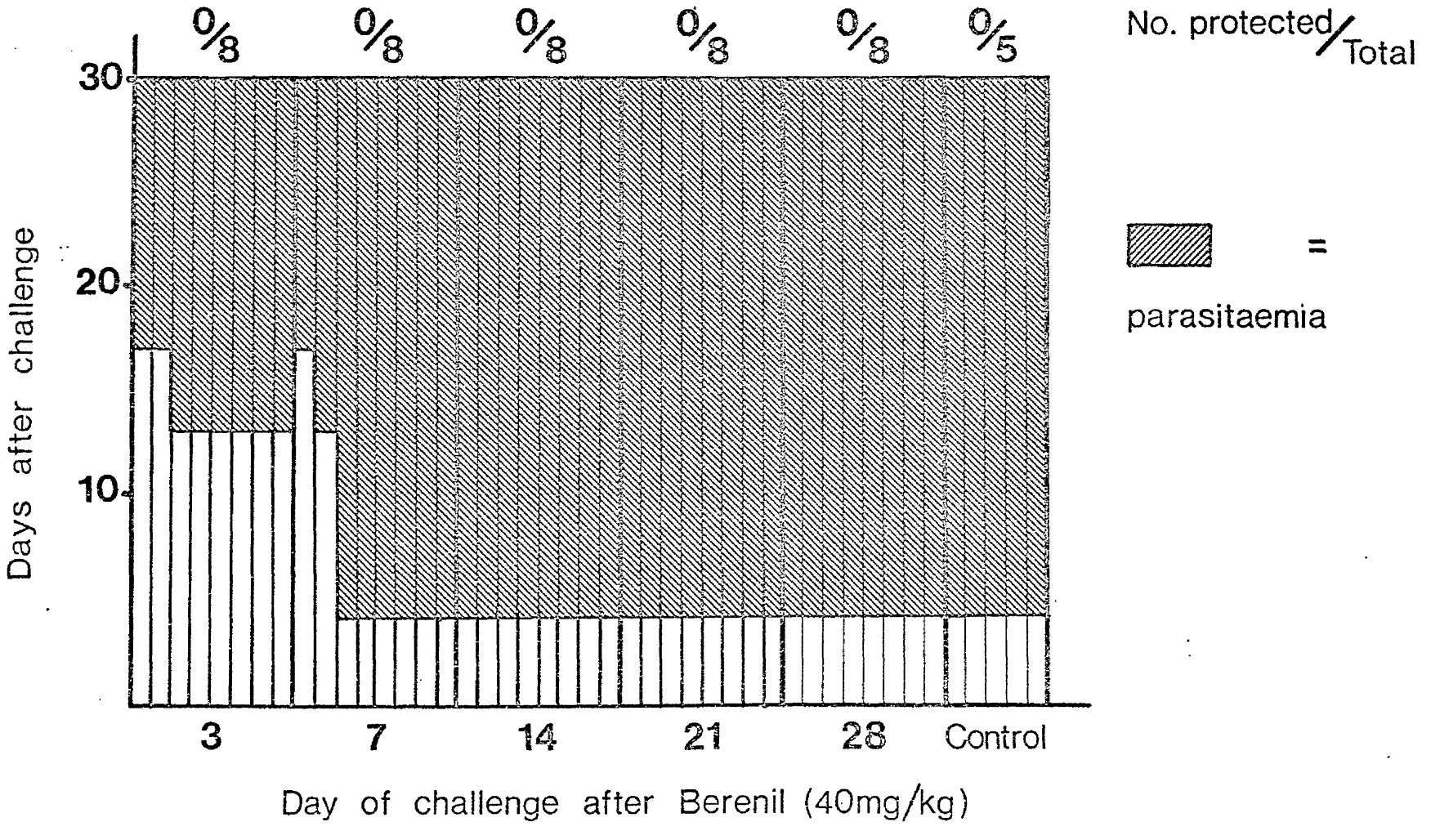


Fig 2.4

Berenil prophylaxis in adult mice.

The appearance of trypanosomes in individual adult mice challenged with T. congolense GVR1 on different days after prophylactic treatment with 40 mg/kg Berenil.

Berenil prophylaxis against T. congolense GVR 1 in C57 mice



can be afforded newborn mice which either receive antibody via the colostrum/milk of an infected mother or are given chemoprophylaxis on the day of birth. The protection given by each is of similar duration. To investigate the response of young mice which receive both antibody and drug, an experiment was done involving 3 series of mice. Series I comprised a group of mothers infected with T. brucei TREU 667/1 whose offspring were left to suckle undisturbed, Series II was a group of normal mothers whose offspring were treated with 40 mg/kg Berenil on the day of birth, and Series III consisted of a group of infected mothers whose offspring also received Berenil prophylaxis on the day of birth. Groups of young mice were then challenged with the homologous parasite at either 16, 23, 31, or 41 days of age (each group comprised a complete litter). A result representative of the numerous occasions this experiment was done is shown in Fig 2.5. As before, young mice which suckle an infected mother are themselves immune for approximately 25 days (5 of 9 mice immune to challenge on day 23, Fig 2.5a), and young mice treated at birth with Berenil resist infection for a similar period (all resistant at 23 days of age, all susceptible at 31 days of age, Fig 2.5b). However, young mice which receive colostrum/milk from an infected mother and also receive Berenil prophylaxis at birth, are able to resist homologous trypanosome challenge for considerably longer. Only 1 of 8 mice was susceptible to challenge on day 31, and on day 41, 3 of 8 mice were completely resistant to challenge, the remaining 5 mice showing prolonged prepatent periods (7-9 days compared with 4 days in controls, Fig 2.5c).

Fig 2.5 Combined immune- and chemo-prophylaxis in newborn mice.

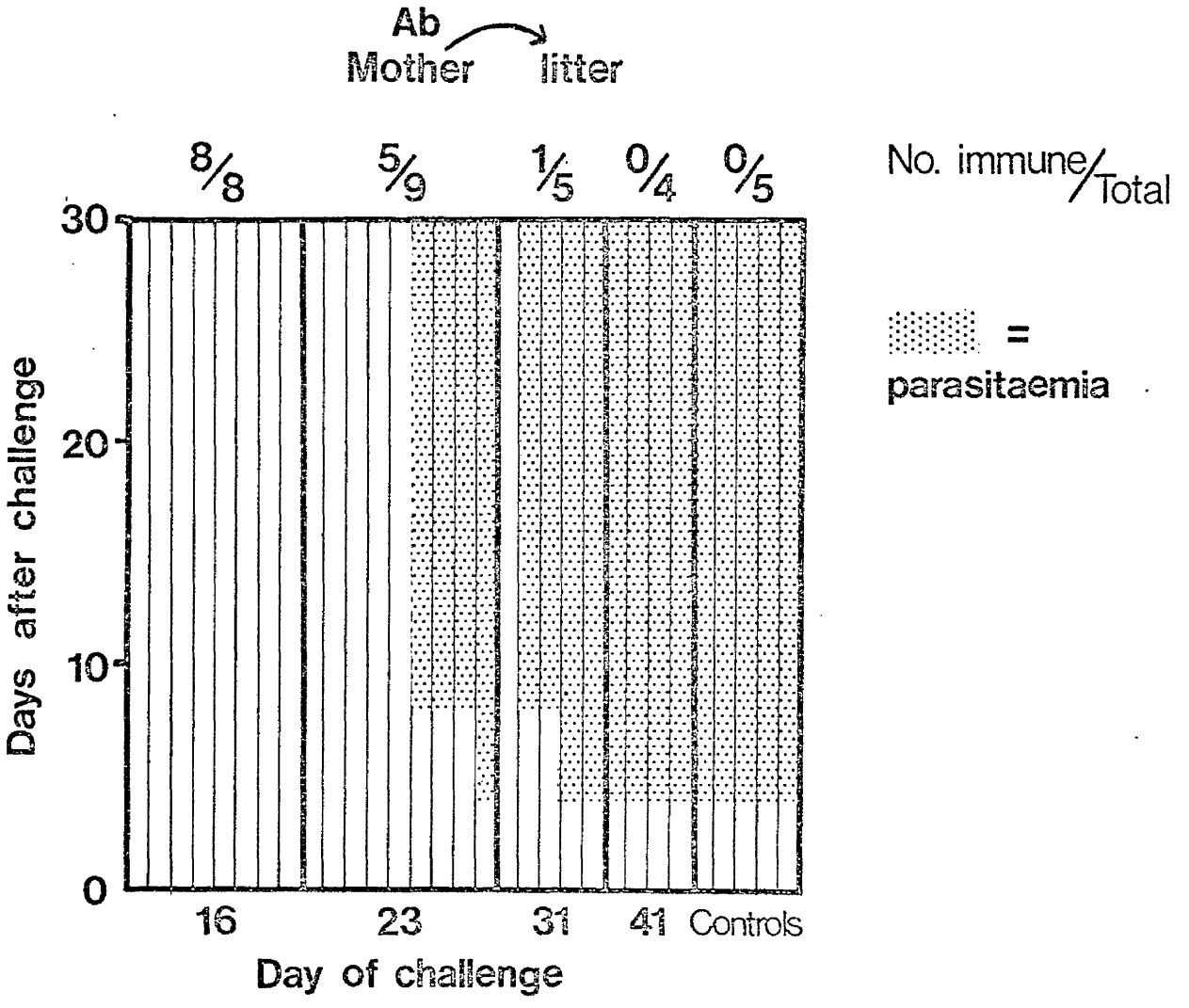
The appearance of trypanosomes in individual young mice challenged with T. brucei TRE 667/1 on different days after birth.

a. Young mice suckling an infected mother.

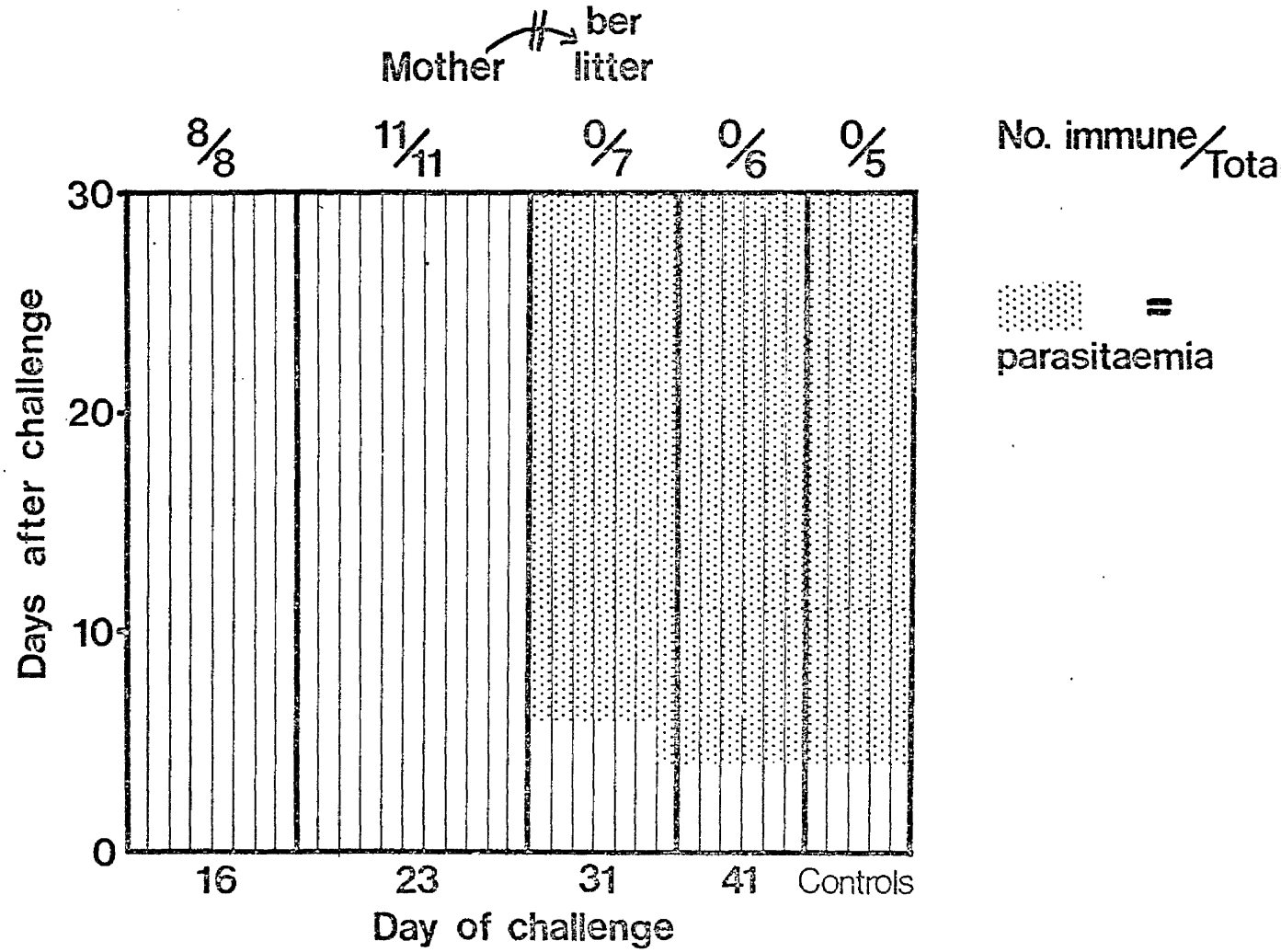
b. Young mice treated at birth with Berenil suckling an uninfected mother.

c. Young mice treated at birth with Berenil suckling an infected mother.

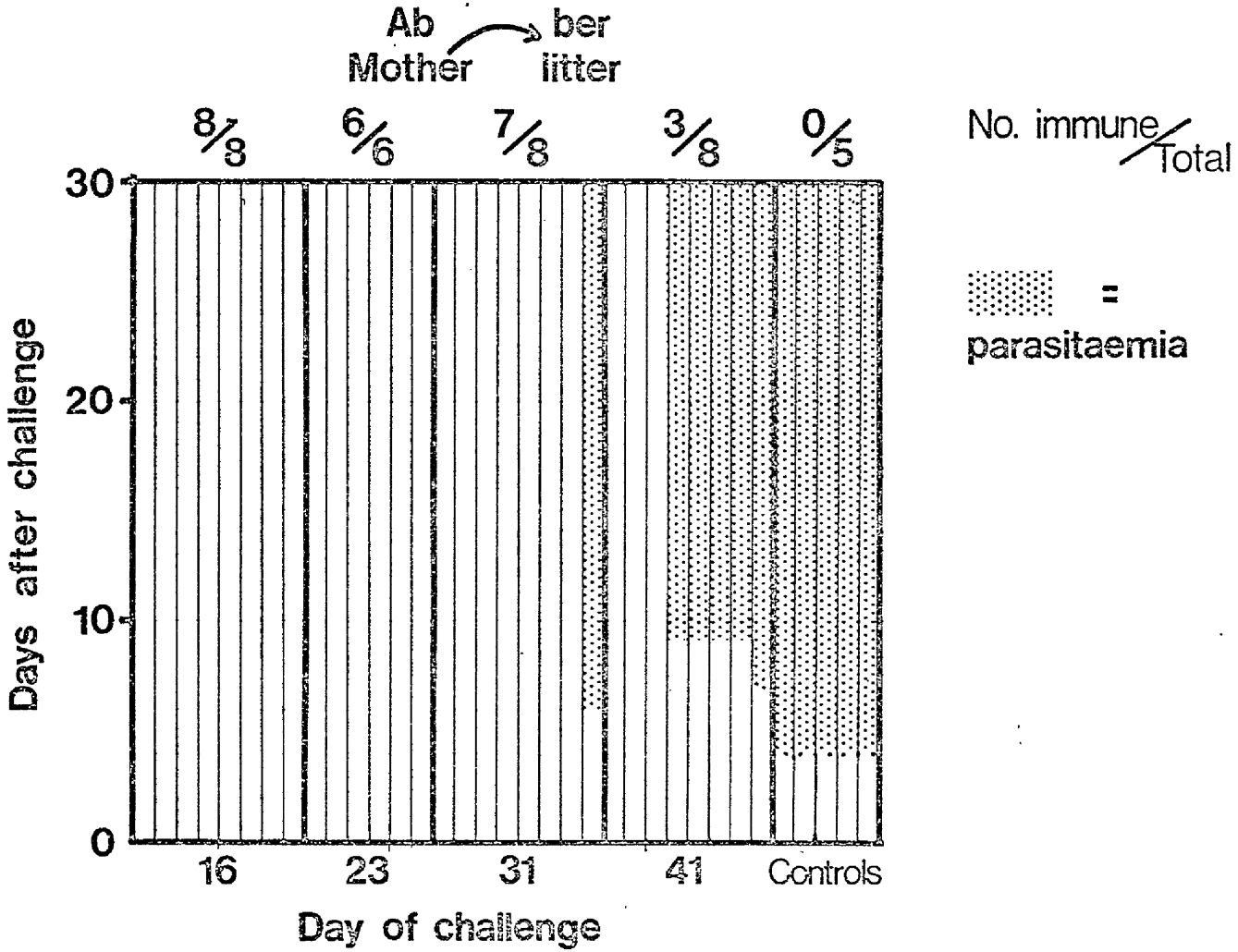
a.


Ab
Mother
litter
8/8
5/9
1/5
0/4
0/5
No. immune/Total
30
20
10
0
Days after challenge
= parasitaemia
16
23
31
41
Controls
Day of challenge

b.


Mother
litter
No. immune/Total
8/8
11/11
0/7
0/6
0/5
30
20
10
0
Days after challenge
parasitaemia
16
23
31
41
Controls
Day of challenge

C.


Ab
Mother
ber
litter
8/8
6/6
7/8
3/8
0/5
30
20
10
0
Days after challenge
16
23
31
41
Controls
Day of challenge
No. immune/Total
= parasitaemia

A more extensive experiment was done to investigate further the conditions under which the observed enhanced protection afforded by Berenil and antibody could be reproduced. A series of 7 groups of mothers and litters were set up as outlined below.

I. A pool of normal mothers with their own litters; 5 offspring to be challenged every 5 days as infection controls.

II. Three groups of normal mothers with foster litters; mothers given Berenil the day before receiving their foster litters. This series investigated the possibility of protective levels of Berenil being transferred to young mice in colostrum/milk.

III. Three groups of normal mothers with foster litters; the offspring were born of mothers which had been treated with Berenil before parturition. This series investigated the possibility of Berenil crossing the placenta into the young mice whilst in utero thus providing subsequent protection after birth.

IV. Four groups of infected mothers with foster litters; maternal antibody controls.

V. Four groups of infected mothers with foster litters; mothers treated with Berenil the day before receiving their foster litters. This series investigated whether or not treatment of the mother's infection affected the degree of immunity transmitted to the newborn suckling her.

VI. Four groups of normal mothers with their own litters; offspring treated with Berenil on the day of birth. This series

constitutes the Berenil prophylaxis controls.

VII. Eight groups of infected mothers with foster litters; offspring treated with Berenil on the day of birth. This is the combined immune-/chemo-prophylaxis series.

The protocol for the experiment and the results are shown in Table 2.6.

As in the first experiment, young mice which had suckled a mother which was infected were immune to challenge for up to 25 days of age (Series IV), whereas normal mice suckling uninfected mothers had no resistance to challenge (Series I). Litters fostered to mothers which had been cured of their infection the day before fostering had a similar, if slightly less resistance to challenge (Series V), 5/9 mice being immune to challenge on day 25 compared to 7/7 in the untreated group (Series IV). Berenil treatment of normal mothers affords no protection to their litters (Series II), indicating that trypanocidal levels of Berenil are not transmitted to suckling infants via colostrum/milk. Similarly, the offspring of mothers treated with Berenil before parturition were unprotected, which eliminates the possibility of drug residue in foetal tissue (Series III). Chemoprophylaxis of between 20 and 25 days was evident in offspring treated at birth with Berenil (Series VI). However, prolonged prophylaxis was seen in mice treated at birth with Berenil which were fostered to an infected mother, therefore acquiring in addition passive immunity via colostrum/milk (Series VII). Over 50% of such mice were resistant

Table 2.6 The influence of Berenil chemotherapy on colostrally-derived immunity in mice

Series		Day of challenge after birth								
		15	20	25	30	35	40	45	50	55
I	M ⟶ 0	0/5*	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
II	Berenil M ⟶ 0	0/7	0/8	0/7	-	-	-	-	-	-
III	Foetal Berenil M ⟶ 0	0/6	0/9	0/6	-	-	-	-	-	-
IV	Ab M ⟶ 0	7/7	5/5	7/7	0/8	-	-	-	-	-
V	Ab + Berenil M ⟶ 0	10/10	8/8	5/9	0/7	-	-	-	-	-
VI	Berenil M ⟶ 0	6/6	9/10	3/10	0/10	-	-	-	-	-
VII	Ab Berenil M ⟶ 0	-	10/10	8/8	5/6	6/7	4/6	3/8	1/8	0/8

*No. of offspring which fail to develop parasitaemia/total in litter

M = Mother; 0 = offspring

Fig 2.6

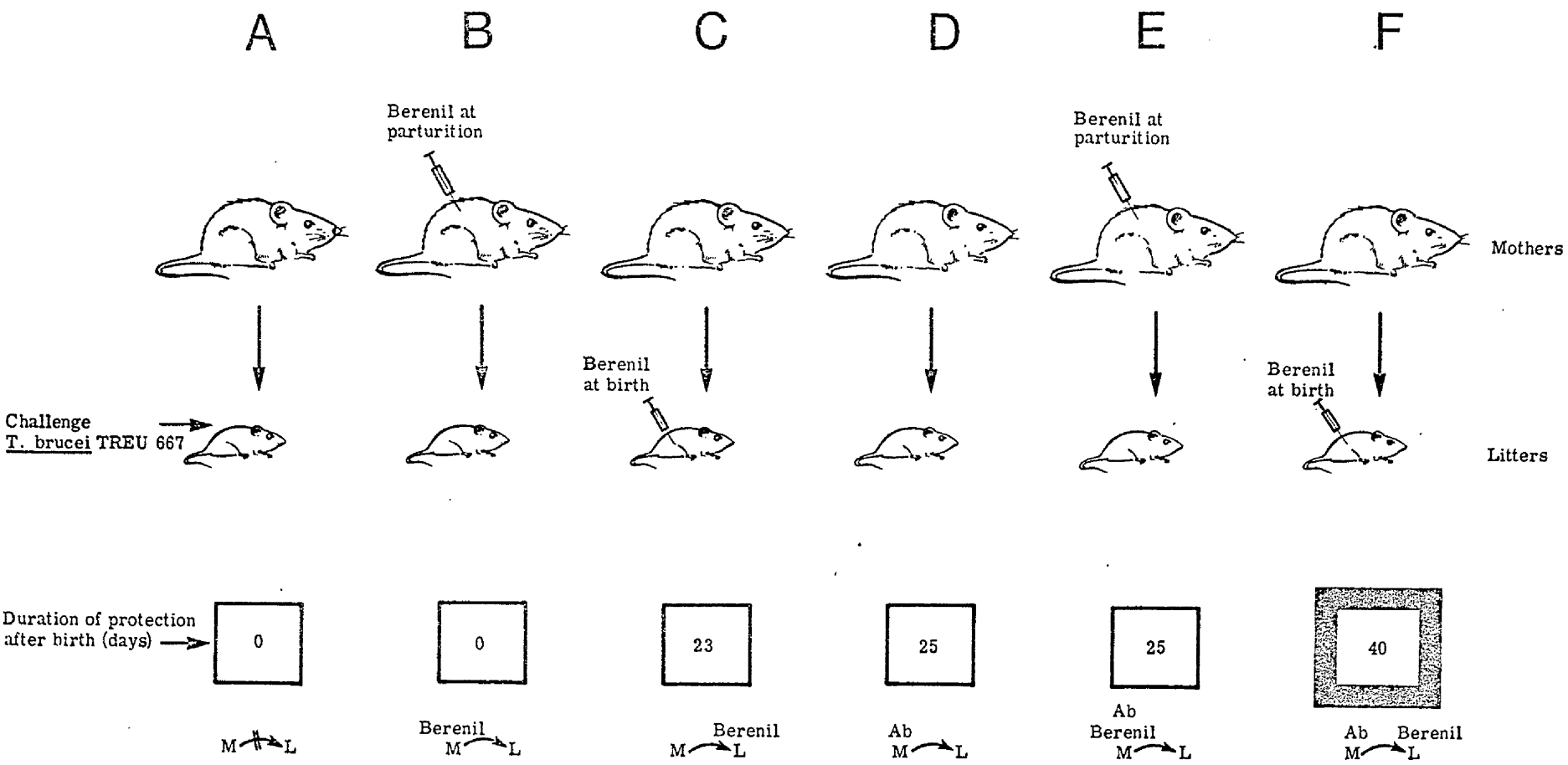
Combined immune-/chemo-prophylaxis in mice to T. brucei TREU 667/1 infection. This figure summarises the findings of numerous experiments, and shows that it is only in a situation in which a neonate receives antibody from a mother, or chemoprophylactic therapy at birth, or both, that there is subsequent resistance to homologous infection. It is only in a situation where both antibody and drug have been received that enhanced resistance results.

Key:

Mothers A, B, and C are uninfected.

" D, E, and F are infected.

All litters are fostered.


A
B
C
D
E
F
Berenil at parturition
Berenil at parturition
Mothers
Berenil at birth
Berenil at birth
Challenge
T. brucei TREU 667
Litters
Duration of protection after birth (days)
0
0
23
25
25
40
M L
Berenil
M L
Berenil
M L
Ab
M L
Ab
Berenil
M L
Ab
Berenil
M L

to challenge at 40 days of age, and complete susceptibility to infection was not achieved until 55 days of age. This compares with the 25 days protection afforded by Berenil treatment alone or maternal antibody alone (Series VI and IV).

Effect of Berenil *in vitro* on infectivity of *T. brucei* TREU 667/1.

The effect of *in vitro* incubation with Berenil on the infectivity of *T. brucei* TREU 667/1 was studied. After incubation for 30 min, trypanosomes were injected into susceptible mice, and monitored for subsequent infection. The result is shown in Table 2.7. At a level in excess of 64 μg/ml, 2.5×10^5 trypanosomes were completely neutralised by Berenil. At 16 and 32 μg/ml Berenil, a proportion of recipient mice became positive, but the prepatent period in these mice indicated that the infectivity of the trypanosome suspension was much less than controls. As the concentration of Berenil decreased further, thus the difference from the control group also decreased.

Table 2.7 The effect of *in vitro* incubation with Berenil on the infectivity of *T. brucei* TREU 667/1

Berenil concentration µg/ml	No. positive/ No. in group	Prepatent period in positive mice (days)
128	0/6	-
64	0/6	-
32	3/6	12,13,14
16	4/6	9,11,13,13
8	6/6	6,6,6,6,8,11
4	6/6	5,5,5,5,5,6
2	6/6	3,4,4,5,5,6
1	6/6	3,3,3,3,4,4
Controls	6/6	3,3,4,4,4,4

DISCUSSION

Early postnatal life in many mammals relies on maternally derived immune protection to combat infection, but the role that this may have in influencing the survival of animals exposed in early infancy to trypanosomiasis, has been the subject of much conjecture. Experimental studies, however (Culbertson, 1938, 1940), had shown that the young of mother rats recently recovered from a *T. lewisi* infection, or mice whose mothers had recovered from a *T. duttoni* infection, were themselves immune for the first few weeks of life. Similarly, a normally fatal consequence of *T. cruzi* in young rats was modified to a mild abortive infection by suckling recovered or passively immunised mothers (Kolodny, 1939).

The findings presented in this chapter demonstrated that immunity to the salivarian trypanosomes *T. brucei* and *T. congolense* can be conferred on young mice by suckling mothers which were experiencing an active trypanosome infection. Moreover, the experiments with *T. brucei* showed that mothers cured of their infection prior to parturition transmitted to their sucklings a similar level of protection as those which were not given trypanocidal therapy.

This immunity is by way of colostrum/milk after birth; effective placental transmission appears not to occur since offspring of infected mothers are susceptible to challenge if suckled with non-immune colostrum. Litters of non-immune mothers, however, are protected if after birth they are suckled by an infected mother (Fig 2.1).

Takayanagi, Takayanagi, Yabu and Kato (1978) reported maternal protection in neonatal rats to T. gambiense infection, but only if the offspring had received colostrum from their own mothers. Rats fostered from control mothers to immune mothers were susceptible to challenge, implicating an essential in utero component of subsequent immunity in neonates, and they concluded that transplacental IgG was more effective than colostrum-derived IgA in protecting offspring against trypanosome infection. However, experimental studies in young rodents have shown that IgM and IgA of maternal origin are not transmitted across the neonatal gut to the circulation (Jones, Hemmings and Williams, 1973; Hemmings, Jones and Williams, 1973). Furthermore, placentation in rodents is of the haemoendothelial type, and while antibody transmission is thought to occur to a small extent before birth, the majority of immune transfer from mother to offspring occurs after birth (McGirr, 1947; Brambell, 1970). In this respect, the mouse or rat is a particularly good model for bovines and ovines, whose syndesmochorial placentation allows materno-foetal transfer of immunoglobulin only after birth by way of colostrum.

Transmission of immunity after birth by way of the neonatal gut involves transport of the immune globulin across the gut wall from the lumen to the circulation without loss of its immunological activity. In young rodents, which receive most of their passive immunity from the mother after birth, milk proteins are taken into the absorptive cells of the gut non-selectively by pinocytosis (Clark, 1959; Vacek, 1964). Only a small proportion

of the absorbed protein is then transmitted into the circulation intact, and most of this is γ-globulin. It is thought that γ-G globulins are transmitted across the gut of suckling rodents by virtue of transmission sites on the Fc regions of the molecules (Brambell, 1966) and the markedly different modes of transmission of homologous IgG_1 and IgG_2 may reflect the operation of distinct receptors for these immunoglobulins. Jones (1974) showed that different regions of the small intestine transported different chromatographic fractions of IgG differently and with varying efficiency, an observation consistent with an uneven distribution of distinct receptors in the gut. Homologous γ-globulins are transmitted more easily than heterologous globulins (Bangham and Terry, 1957), and the efficiency of transmission of heterologous γ-G-globulin observed in neonatal feeding experiments, presumably reflects the degree of resemblance of its Fc regions to those of the homologous protein (Morris, 1969, 1976; Jones, 1976).

The main site of transport in rat and mice is the small intestine. Evidence of transmission has been shown in the ileum (Bamford, 1966) and cytological evidence supports this and also suggests that the jejunum is also involved (Clark, 1959; Kraehenbuhl, Gloor and Blanc, 1967). More recent evidence suggests that the proximal rather than the distal half of the small intestine is the principal site, where entry into the cells occurs by selective surface endocytosis (Rodewald, 1970, 1973; Morris and Morris, 1976). There is no evidence that transport occurs in the duodenum or the stomach. However, there can be no doubt as to the efficiency of intestinal transmission of immunoglobulin, since significant

quantities of maternal IgG pass into the circulation of suckling mice during the first 15 days of life despite the low concentration of IgG in the mother's milk (less than 5% of the maternal serum concentration, Stechschulte and Austen, 1970).

The termination of the capacity to transmit immunoglobulin from the intestine to the circulation occurs comparatively suddenly in the mouse between 14 and 16 days of age and in the rat between 18 and 20 days (Halliday, 1955 , 1959). In a normal 17 day old mouse and the normal 21 day old rat there is no detectable transmission. The means by which this change in the permeability of the gut occurs is still unclear, but it has been shown that replacement of the vacuolated cells of the distal intestine which take up polyvinylpyrrolidine (PVP) by a new cell type which is PVP impermeable, a clearly defined feature of the maturation of the small intestine which occurs in rodents at this time, can be induced by certain steroids (Daniels, Hardy, Malinowska and Nathanielz, 1973). Treatment with certain steroids will also induce precocious closure to antibody and labelled globulin (Halliday, 1959; Jones, 1972; Morris and Morris, 1974) and it is thought that such treatment must also affect those proximal and middle regions of the intestine which are actively engaged in immunoglobulin transmission and whose maturation is thought to be under natural control by secretions from the adrenal cortex (Daniels et al, 1972).

The development of immune competence in rodents suckling immune mothers is complicated by interference of the induction of

active immunity through the presence of antibody of maternal origin (Solomon, 1971). Halliday (1968) showed that anti-B. abortus agglutinins of maternal origin, even at very low concentrations, can significantly inhibit the active production of agglutinins in young rats, but the effect is transient because of the decline in the passive immunity and the increasing potential for active immunity. This interference is due to competition for antigen between specific receptors on lymphocytes and antigen-combining sites on molecules of circulating antibody derived from the mother (Leiper and Solomon, 1976). If maternal antibodies are present in sufficient concentration, they complex with antigen which is then removed so rapidly from the circulation by phagocytes that no antigenic stimulation occurs. The amount of opsonic activity is finite, and provided the antigen can remain in the circulation long enough to combine with specific receptors on the lymphocytes of reactive clones, successful induction of the immune response may occur (Leiper and Solomon, 1976).

The result of the antigenic priming experiment showed that while maternally-derived antibody is no longer protective by 33 days of age (Table 2.5, Group B) animals primed at 16 days of age, when gastrointestinal transmission of immunoglobulin is ceasing (Halliday, 1955, 1959) but maternally derived antibody is still protective, can successfully respond to a subsequent challenge 17 days later (group A). Thus it appears that while a challenge infection at day 16 is eliminated, presumably by hepatic phagocytosis (Table 2.4), induction of the immune response occurs.

Since maternal IgM and IgA are not transmitted across the neonatal gut (Jones et al, 1973; Hemmings et al, 1973), the newborn mouse relies on colostrum-derived IgG for immunity, and within the IgG class, IgG_2 is transmitted more readily than IgG_1 (Morris, 1969, 1976). Assuming a half-life of 4 days in mice for maternally-derived IgG (Fahey and Sell, 1965) very low levels exist in sera by 24 days of age (Brambell, 1970), which is around the time that young mice become susceptible to infection with T. brucei (Fig 2.1). Thus, in the absence of antigenic stimulus, mice of this age will no longer have protective levels of maternally-derived anti-trypanosome antibody, and succumb to challenge with T. brucei. Presumably, the shorter period of protection afforded against T. congolense is a reflection of a lower level of antibody having been transmitted to the offspring at the time of termination of absorption.

It is interesting to note that congenital infections of trypanosomiasis have been reported on a number of occasions (Olowe, 1975; Burke, Bengosi and Diantete, 1974; Buyst, 1976), and it has been claimed (Lanfranchi, 1915) that infection can be transmitted from mother to offspring in the milk, though this has been disputed (Werner, 1954). No evidence of vertical transmission of infection from mother to offspring was found in the present study.

The observations on the prophylactic activity of Berenil against T. brucei confirm those of previous studies that "biologically-active" Berenil, which is presumably retained in the tissues, has a protective effect lasting several weeks (van Hoeve and Cunningham,

1964; Lumsden, Herbert and Hardy, 1965; Raether, Hajdu, Seidenath and Damm, 1972; Zahalsky and Weinberg, 1976; Williamson, 1976). Diamidines related to Berenil (eg pentamidine and stilbamidine) have been shown to bind to nucleoproteins (Kopac, 1947; Makulu and Waalkes, 1975), which may facilitate tissue retention. However, no prophylactic activity was seen against T. congolense, an observation in agreement with that of Fairclough (1963) and Fink and Dann (1974).

Newborn and adult mice are resistant to infection for approximately 25 days after treatment with 40 mg/kg Berenil (Fig 2.2, 2.3). Furthermore, it is of interest to consider that between birth and 25 days of age, a mouse increases its bodyweight by 10-fold, thus reducing, in absolute terms, a dose of 40 mg/kg Berenil at birth to 4 mg/kg. Such a dose is prophylactic in adult mice for a period of only 7-14 days.

Immunity in calves exposed to natural tsetse challenge has been reported on several occasions. Chandler (1952) noted a calf alive and uninfected at 8 weeks of age, despite its mother being infected and there being a constant exposure to tsetse. Calves of cows newly introduced into an endemic area developed trypanosomiasis within 5 weeks of birth (Whiteside, 1962). Those born 8 months later took an average time of 14 weeks, and calves born 12 months later did not become infected until 30 weeks of age. Furthermore, it is known that offspring of game animals of species which thrive in tsetse country succumb to trypanosomiasis if exposed to infection for the first time when they are adult (Fiennes, 1970) which

strongly suggests a situation in which maternally-derived immunity has waned and antigenic priming has been absent (vide supra). The same author also reported a situation in Kenya which illustrated the ability of calves, born of dams with experience of trypanosomiasis resulting from light exposure to tsetse, to resist or tolerate infection with pathogenic trypanosomes.

Drager and Mehlitz (1978) noted significant age-related resistance in 416 buffalo in Botswana. In calves of less than one year of age, trypanosomes were found in only 1 of 74 animals, whereas the prevalence of parasites in adult animals was much higher (31 of 90 animals aged between 1 and 3 years were positive for trypanosomes). They concluded that maternally-derived immunity may have been an influencing factor in these findings.

Fiennes (1953) investigated calf infections in the course of experiments on the maintenance of cattle protected with Antrycide prosalt. All of the treated cows produced normal healthy calves, and the bull calves which received no treatment remained "more or less refractory to infection". In the same context, commenting on the observations of Whiteside (1962), Murray and Urquhart (1977) stated that since the dams received trypanocidal therapy intermittently, the resistance of the newborn may have been through drug residues in foetal tissue or milk, rather than a genuine maternally-derived immunity. The current study, however (Table 2.6, Fig 2.6), indicates that newborn mice acquire no or insufficient drug either whilst in utero (Series III) or in the milk (Series II) to resist infection.

Attempts to produce immunity in animals against African trypanosomiasis have been undertaken by several investigators with varying degrees of success. As early as 1904, Ehrlich and Shiga demonstrated a high level of immunity by infecting an animal with a given strain of trypanosomes and then curing it with a trypanocidal drug. These animals proved extremely resistant to challenge with the homologous trypanosome strain.

Mice have been successfully immunised against homologous challenge using Berenil-treated trypanosomes (James, 1976), and the results of Kinnamon and Rane (1978) confirmed the reports of others (Soltys, 1955; Fulton, 1944; Fiennes, 1950b; Wilson, Cunningham and Harley, 1969) that prophylactic protection may be enhanced by frequent test challenges with trypanosomes, although they did not support the contention that neither drug therapy nor immunization alone could be as effective a means of protection as when they are given in combination (Fiennes, 1950).

Such a situation, however, seems to be the case in the present study. A combination of chemoprophylaxis at birth and ingestion of anti-trypanosome antibody from the mother, markedly extends the period that young mice are refractory to infection beyond that which is afforded by chemoprophylaxis or immuno-prophylaxis alone (Table 2.6, Fig 2.6).

The explanation for this is unclear. Berenil acts by combining with and preventing the replication of DNA. It resembles certain phenanthridine and acridine drugs in being able to selectively block kinetoplast DNA replication, but its trypanocidal

activity is more likely due to its action on nuclear DNA. It has been shown to bind rapidly and irreversibly to trypanosomes (Bauer, 1958), and at low doses of Berenil, the kinetoplast alone seems to form Berenil/DNA complexes (Newton and Le Page, 1967). Fink and Dann (1974) showed that diamidines concentrated at higher levels in the tissues than in plasma, perhaps through their facility of binding to nucleoproteins (Makulu and Waalkes, 1975) and were slowly eliminated.

Thus one may envisage a situation which in mice occurs around 25 days of age (vide supra) in which levels of Berenil and maternal antibody per se have fallen below that which affords protection to challenge, but in concert can act in a trypanocidal manner. Trace amounts of Berenil may be able to interfere sufficiently with the parasite to slow or even stop replication, or alternatively allow replication but prevent antigenic variation. However transient such an effect may be, it may enable a small amount of antibody, which alone could not cope with a challenge infection, to eliminate the parasite. In vitro incubation of Berenil and trypanosomes, with subsequent injection into susceptible mice (Table 2.7), showed that at low levels of drug, the infectivity of the trypanosomes was markedly reduced, but not abolished. The occurrence of a similar effect in vivo with tissue residues of Berenil would support the argument forwarded above of a transient deleterious effect of the drug on the challenge inoculum. This may be compared with the conclusion of Fiennes (1953) that the effectiveness of a drug depended mainly on its

ability to suppress infection to a point where the animal's own defence mechanisms were able to gain control.

A similar effect was recorded by Goodwin and Tierney (1977) who observed higher trypanocidal titres in the plasma of rabbits which had been infected and treated with drug than in those receiving drug alone, and ascribed this to a boost in the antibody response of the host through the release of additional antigenic protein by chemotherapy. Such a mechanism cannot be operable here, however, since the young mice have no experience of trypanosome antigen until the day of challenge.

The results of the experiments in this chapter offer a possible approach to augmenting immunity in the field. If it transpires that within particular locations the number of serodenes is limited and that the antigenic nature of metacyclic trypanosomes is a stable characteristic (vide supra), production of immunity in dams by "cocktail" vaccination may be possible. This has been done with a mixture of VATs in mice (Herbert and Lumsden, 1968) and cattle (Scott, Holmes, Jennings and Urquhart, 1978) though broke down when there was present in the challenge inoculum a VAT not represented in the vaccine. Prophylactic therapy in newborn in conjunction with the acquisition of passive immunity through immune colostrum may be of practical value in field situations in the generation of resistance to the disease.

CHAPTER III

Genetic resistance of mice to *T. congolense* infection

INTRODUCTION

The exploitation of genetic resistance to disease is becoming an increasingly important consideration in livestock development programmes, particularly where conventional disease control measures are not effective or are too costly, and consequently the application of such an approach to African animal trypanosomiasis has provoked much interest in recent years.

Despite the use of many control strategies in the past, there has been little effect on the disease at the continental level. There are several factors responsible for this. First, there are 22 species of tsetse capable of transmitting infection; these are adapted to a wide range of habitats, thereby contributing to the widespread nature of the disease. Secondly, the 3 trypanosome species pathogenic for domestic livestock, Trypanosoma congolense, T. vivax and T. brucei exhibit a wide host range for both domestic and wild animals. Thirdly, the phenomenon of antigenic variation which leads to persistent parasitaemia provides an excellent opportunity for transmission of infection by tsetse. At the same time, the implementation of current control measures poses several problems. The use of drugs, both therapeutically and prophylactically, can be costly because repeated treatments are required and diagnostic facilities are necessary if the drugs are to be used properly. Furthermore, frequent use or misuse can lead to the development of chemo-resistance, a risk compounded by the fact that the number of

drugs commercially available at present is extremely limited. Tsetse control followed by eradication has been successful in certain regions, but as with drug strategies, the cost is high and it is essential that the eradicated area is kept under rigorous surveillance for several years and protected by man-made tsetse barriers to prevent reinvasion. In addition, the emotive question of environmental hazards created by the use of the insecticides arises.

In the face of these problems, increasing attention has been focused on the potential use of genetically resistant or trypanotolerant livestock. There is no clear definition of trypanotolerance. At one extreme, Pagot (1974) has defined trypanotolerance as a "racial aptitude (of cattle) to maintain themselves in good condition and to reproduce while harbouring trypanosomes without showing clinical signs of the disease". He recommended that they be introduced widely in high tsetse challenge areas throughout Africa. At the other end of the scale, Stephen (1966) stated that "tolerance is far from absolute" and concluded that propagation of trypanotolerant breeds was not to be recommended as a satisfactory means of improving the supply of protein in the densely populated areas of West Africa. These conflicting views reflect the complexity of the problem, since both statements are true for the circumstances under which each author made his observations.

With regard to the economic potential of trypanotolerance, more information is required on several aspects of the trait before

widespread exploitation of trypanotolerant breeds can be recommended (Stewart, 1951; Chandler, 1952). It is important to know the extent of the differences in resistance between different breeds and also within the same breed living under different levels of trypanosomiasis risk, under different management systems in different ecological zones. At the same time, an understanding is required of the mechanism(s) underlying trypanotolerance, the genetics of heritability, and the stability of the trait, ie, how it is affected by environmental factors.

The lack of suitable herds for study and the genetic heterogeneity of domestic livestock have made it difficult to analyse the mechanisms and inheritance of trypanotolerance. Accordingly, this chapter describes a series of experiments to investigate the susceptibility of different strains of mice to T. congolense infection, and attempts to establish whether trypanotolerance is related to a modification of the pathophysiological changes characteristic to infection or can be explained in terms of differences in the efficacy of the immune response to the parasite.

MATERIALS AND METHODS

Mice. These were CFLP and C57 B1/6 female mice, aged 6-8 weeks unless otherwise stated. The supplier of these mice has been detailed previously (p.36). BALB/C, CBA, C3H/He, Porton and NZB mouse strains were also supplied by Anglia Laboratory Animals, C57B1/B.10D2 came from MRC (Carshalton), C57B1/ 10Sc.Sn from Olac, 1976 Ltd, and C57B1/ B from the breeding colony of the Zoology Department, Glasgow University. All mice, as soon as infected, were given oxytetracycline (Terramycin Soluble Powder, Pfizer Ltd) in their drinking water for the duration of the experiment. All experiments were conducted with a minimum of 5 mice of each strain.

Parasites. The histories of T. congolense GVR1 and T. brucei TRPU 667 are outlined in Appendix I. T. congolense GUP 93 was a gift from Professor K. Vickerman, Department of Zoology, Glasgow University; LUMP 52, 79, 89 and 588 (all T. congolense) were supplied by Professor W.H.R. Lumsden, London School of Tropical Medicine and Hygiene, and T. congolense LR1 was provided by Professor M.J. Clarkson, University of Liverpool Veterinary School. These stabilates were passaged in mice irradiated at 650 Rad one day before infection, the blood removed at peak parasitaemia, and preserved in liquid nitrogen. Unless stated otherwise, each mouse was infected by (i.p.) injection with 1×10^4 trypanosomes, prepared from the frozen stabilates in PSG, pH 8.0.

Estimation of parasitaemia and haematological values. Tail blood

from the mice was examined for trypanosomes by the wet film technique, and approximate parasite counts were done by a matching method (Herbert and Lumsden, 1976). Parasite numbers were checked at critical points of the parasitaemic profile by performing counts in an Improved Neubauer Haemocytometer chamber (Hawksley and Sons Ltd., Lancing, Sussex). For packed red cell volumes (PCV), mice were bled from the tail vein into microhaematocrit tubes containing heparin as anticoagulant, and centrifuged (Hawksley). Plasma samples, when required, were obtained either a) by cutting such capillaries with a diamond cutter just above the buffy coat, giving 25-40 µl of plasma, or b) by killing mice under deep trichloroethylene anaesthesia, resulting in terminal plasma samples of approximately 0.5 ml volume. These were stored at -20°C in the sealed capillary or plasma vial until required.

Radioisotopic techniques.

a) Labelling methods. Radiolabelled parasites were prepared by the *in vivo* method using ^{75}Selenomethionine described in Chapter 4, and the tissue distribution of radiolabelled parasites determined as described (p. 166).

Red blood cells were labelled with ^{51}Cr by incubating blood from uninfected mice of the same strain with ^{51}Cr as sodium chromate (Radiochemical Centre, Amersham) and heparin as anticoagulant for 30 min at 37°C. After washing twice with PBS, pH 7.4, the cells were prepared for injection by reconstitution in a suitable volume of PBS. Each mouse received approximately 20 µCi of ^{51}Cr-labelled cells (Jennings, Murray, Murray and Urquhart, 1974).

Mouse albumin (Sigma Chemical Co. Ltd., London) was labelled with ^{125}I (Radiochemical Centre, Amersham) by a modification of the iodine monochloride method (McFarlane, 1958).

b) Trypanolysis of ^{75}Se-T. congolense. ^{75}Se-labelled T. congolense, 1 x 10^8 organisms in 0.1 ml, were incubated with serum or plasma (0.2 ml) from C57Bl or CFLP mice together with 0.2 ml guinea pig serum (0.2 ml) for 2 hours at 37°C. To achieve maximum release of ^{75}Se-activity, 0.2 ml of MP.40 (10%) or deoxycholate (20%) was used in the place of test serum. Minimum ^{75}Se-release was assessed from ^{75}Se-labelled parasites incubated in PSG or normal mouse serum. Each test was carried out in triplicate.

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

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

c) Red cell survival. This was measured in groups of 10 infected and 10 control mice using ^{51}Cr red cells over a period of 10-13 days following infection in i) both strains (ie C57Bl/6 and CFLP) from the day of infection, and ii) C57Bl/6 mice 9 weeks post-infection. After injection of the radiolabelled erythrocytes, blood samples were taken at 5 min post-injection and then at 2 day intervals, using a 25 µl microcapillary tube (Drummond Scientific Co., U.S.A.). Each tube and contents was inserted into a counting

vial containing 5 ml 0.02N NaOH, and the sample counted on a multichannel gamma scintillation counter (Packard Tri-Carb, Chicago) for enough time to give sufficient counts for analysis. The radioactivity of the samples was expressed as counts/min/ml packed red cells and plotted as a percentage of the 5 min sample against time on semi-logarithmic paper. Plotted values are the means of 10 animals in each group on any given day, and disappearance curves are "best fit" lines calculated by regression analysis on the group results.

d) Plasma and red cell volumes. These were determined in groups of mice of both strains by application of the dilution principle at 8 days post-infection using ^{51}Cr red cells and ^{125}I albumin. The two were injected together into a tail vein and blood samples were taken at 5, 15, 30, 40 and 60 min post-injection.

The radioactivity of the red cells and plasma as counts/min/ml were plotted on semi-logarithmic graph paper and the zero time values obtained by regression analysis. The red cell and plasma volumes were determined by dividing the respective total injected activities by these values.

Blood biochemistry determinations. These were carried out on fresh, unfrozen samples of serum within 3 hours of collection from mice on the eighth day of infection and at suitable intervals thereafter. Lactate dehydrogenase, creatine phosphokinase, aspartate transaminase and alanine transaminase were measured on a reaction rate analyser (L.K.B. Products, Bromma, Sweden), using commercial test kits (Boehringer, Mannheim, Germany).

Total protein, albumin, creatinine, alkaline phosphatase, urea, glucose, bilirubin and phosphate were measured on an Auto-analyser II (Technicon Instrument Corporation, New York). Ca^{++} and Mg^{++} were estimated by atomic absorption (Pye Unicam, Cambridge, England), Na^{+} and K^{+} using a flame photometer and chloride on a chloride meter (Evans Electroselenium Ltd., Essex, England).

Immunoglobulin estimation. Serum levels of IgG_1, IgG_2 and IgM were quantified by radial immunodiffusion (Mancini, Carbonara and Heremans, 1965) using antisera against mouse IgM and IgG prepared in rabbits (Nordic Immunochemicals, Berkshire).

Humoral response to horse red blood cells. At 7, 14, 21, 42, 84 and 120 days after infection mice were injected i.p. with 1×10^9 horse red blood cells (HRBC) which had been washed three times with PBS. Five days later the mice were killed and serum collected. This was inactivated at $56^{\circ}C$ for 30 min and duplicate doubling dilutions were prepared with 25 µl of PBS in microtitre plates (Gibco Biocult Laboratories Ltd., Paisley, Scotland). To each well was added 25 µl of a 2% suspension of washed horse red blood cells (HRBC). The plates were then covered and left overnight at room temperature after which the haemagglutination titres were read as being the highest dilution of serum giving complete agglutination.

Trypanosome infectivity neutralization test. This test for antibody is based on the incubation of serum from infected mice

with homologous trypanosomes and the subsequent inoculation of the mixture into normal mice. Absence of a subsequent parasitaemia in these mice is taken as evidence of antibody (Lumsden, Herbert and McNeillage, 1973). Two pools of serum were obtained 10 days post-infection from groups of mice of both strains and tested for their infectivity neutralization capacity. Each test well contained 200 µl serum, 50 µl guinea pig serum (complement) and 5×10^4 trypanosomes in 250 µl PBSG. After 30 min at $4^{\circ}C$ the contents of the test wells were taken up in a syringe and 12 normal CFLP mice were each inoculated with an aliquot estimated to contain 1×10^4 trypanosomes. The mice were then monitored regularly for the appearance of parasites by wet blood film examination for 30 days.

Irradiation of mice. Where required, mice were subjected to total body irradiation (650 rad) in a ^{60}Co source one day prior to infection.

Drug suppression of mice. This was done using the agent cyclophosphamide (Koch-Light) at a dose of 300 mg/kg body weight 3 days prior to infection.

Splenectomy. Mice were splenectomised under trichlorethylene anaesthesia 14 days before being used in experiments. After the fur had been removed by shaving and disinfection of the skin with Savlon (ICI) followed by 70% ethanol, a small incision was made over the clearly visible spleen. The peritoneal wall was cut

and opened by blunt dissection, and the spleen externalised with blunt forceps. The spleen's venous and arterial blood supply was cut off with a tight suture tied around the pedicle, and the organ was excised. The peritoneal wall was closed with 2 silk sutures and the abdominal skin closed using 2 suture clamps.

Activation of the Mononuclear Phagocytic System. The following immunostimulants were used:

i) Escherichia coli lipopolysaccharide (phenol extract, Sigma Chemical Co., London). Mice were given 10 μg LPS i.v.

ii) Bordetella pertussis. This was a gift from Professor A.C. Wardlaw, Microbiology Department, Glasgow University, and each mouse received an i.v. injection of 5×10^9 organisms.

iii) Bacillus Calmette-Guerin (BCG vaccine, Glaxo, Greenford). Mice received an i.v. injection of 1.5×10^7 organisms per mouse.

iv) Corynebacterium parvum (Coparvax, Wellcome Ltd., Kent). 0.2 ml was injected either i.v. or s.c. This corresponds to 1.4 mg dry weight of organisms.

v) Freunds complete adjuvant (Difco Laboratories, USA). 0.2 ml was injected i.p. into each mouse.

vi) Levamisole hydrochloride (Nemicide, ICI, Cheshire) was injected i.v. or s.c. at a level of 75 μg/mouse.

E. coli LPS, B. pertussis, CFA and levamisole were all given on the day of infection. BCG was given either on the day

of infection or on days -28, -14, -11, -7, or -1. One group of mice received two doses on day -11 and day -1. C. parvum was injected on the day of infection or 14 days previously.

Irradiation of trypanosomes. Trypanosomes were exposed to gamma-irradiation to destroy their infectivity. This was done in a ^{60}Co source at a dose of 60,000 rad. Trypanosomes were maintained in ice in PSG containing 10% Foetal Calf Serum during irradiation.

Statistical analysis. Group results are presented as mean $\pm$ standard error (S.E.).

RESULTS

Susceptibility of different strains of mice. Ssenyonga (1974) reported that C57Bl mice survived a T. congolense infection for longer than CBA mice. The results of a survival study on a variety of inbred and outbred mouse strains is shown in Table 3.1. After infection with 1×10^5 T. brucei TREU 667, there was a marked similarity in survival time between C57Bl, CFLP, CBA and Porton strains, and although more susceptible, C3H and NZB mice withstood the effects of infection for up to 5 weeks. However, after infection with T. congolense, apart from the NZB which survived for approximately 5 weeks, no strain was able to approach the tolerance to infection shown by the C57Bl strain which lived for over 10 weeks. All other strains succumbed to infection within 3 weeks.

Survival of C57Bl and CFLP mice to different T. congolense strains. In order to confirm that the marked difference in susceptibility of C57Bl and CFLP mice, from above, was not merely a property of this particular parasite in these mouse strains, 3 derivatives of the same stock, GUP 93, GVR 1 and GVR 2 and 5 unrelated stocks LUMP 52, 79, 89, 588 and LR 1 were compared for their effect on C57Bl and CFLP mice. The results (Table 3.2) show a clear trend, despite the necessarily small experimental numbers (5 per group). The extreme virulence of T. congolense LR 1 and LUMP 52 resulted in virtually 100% mortality in both strains of mouse by day 10,

Table 3.1. Mean survival time of mice infected with *T. brucei* and *T. congolense*

	Infected with *T. brucei* TREU 667		Infected with *T. congolense* GVR 1	
Strain of mouse	Number of mice	Days ± S.E.	Number of mice	Days ± S.E.
C57Bl	20	53.7 ± 1.2	49	73.7 ± 8.2
CFLP	20	62.8 ± 4.8	133	14.3 ± 2.0
CBA	20	54.5 ± 3.2	23	9.0 ± 0.4
C3H/He	2-	26.8 ± 0.8	26	14.9 ± 2.0
Porton	20	59.0 ± 5.3	25	21.8 ± 3.2
NZB	8	33.1 ± 2.8	17	37.3 ± 4.3
BALB/c	n.d.	n.d.	12	7.0 ± 0

Table 3.2. Survival of CFLP and C57Bl mice infected with various strains of T. congolense

	Total survivors by day :					
CFLP	10	15	20	30	50	70
GVR 1	0/5	-	-	-	-	-
GVR 2	3/5	0/5	-	-	-	-
GUP 93	5/5	2/5	1/5	1/5	0/5	-
LUMP 52	0/5	-	-	-	-	-
LUMP 79	5/5	2/5	0/5	-	-	-
LUMP 89	5/5	3/5	0/5	-	-	-
LUMP 588	5/5	3/5	2/5	2/5	2/5	0/5
LR 1	0/5	-	-	-	-	-
G57Bl						
GVR 1	5/5	5/5	5/5	4/5	3/5	3/5
GVR 2	5/5	5/5	4/5	4/5	4/5	4/5
GUP 93	5/5	5/5	4/5	3/5	3/5	3/5
LUMP 52	0/5	-	-	-	-	-
LUMP 79	5/5	5/5	5/5	5/5	5/5	5/5
LUMP 89	5/5	5/5	5/5	5/5	5/5	5/5
LUMP 588	5/5	5/5	5/5	5/5	5/5	5/5
LR 1	1/5	0/5	-	-	-	-

whereas a marked superior resistance to infection was shown by C57Bl with each of the other stabilates. It is of interest to note the varying survival in CFLP mice infected with GUP 93, GVR 1 and GVR 2, the latter 2 being derivatives of GUP 93, which during passage appear to have increased in virulence.

Susceptibility of different sub-strains of C57Bl mice to infection. This was done to investigate the influence of genotype within the C57Bl strain on the susceptibility to infection. C57Bl/6 (used above) was compared with C57Bl/TB, C57Bl/B.10/D 2. and C57Bl/10Sc.Sn. Ten CFLP mice served as susceptible controls. No difference was found in survival with T. congolense GVR 1 infection (Table 3.3).

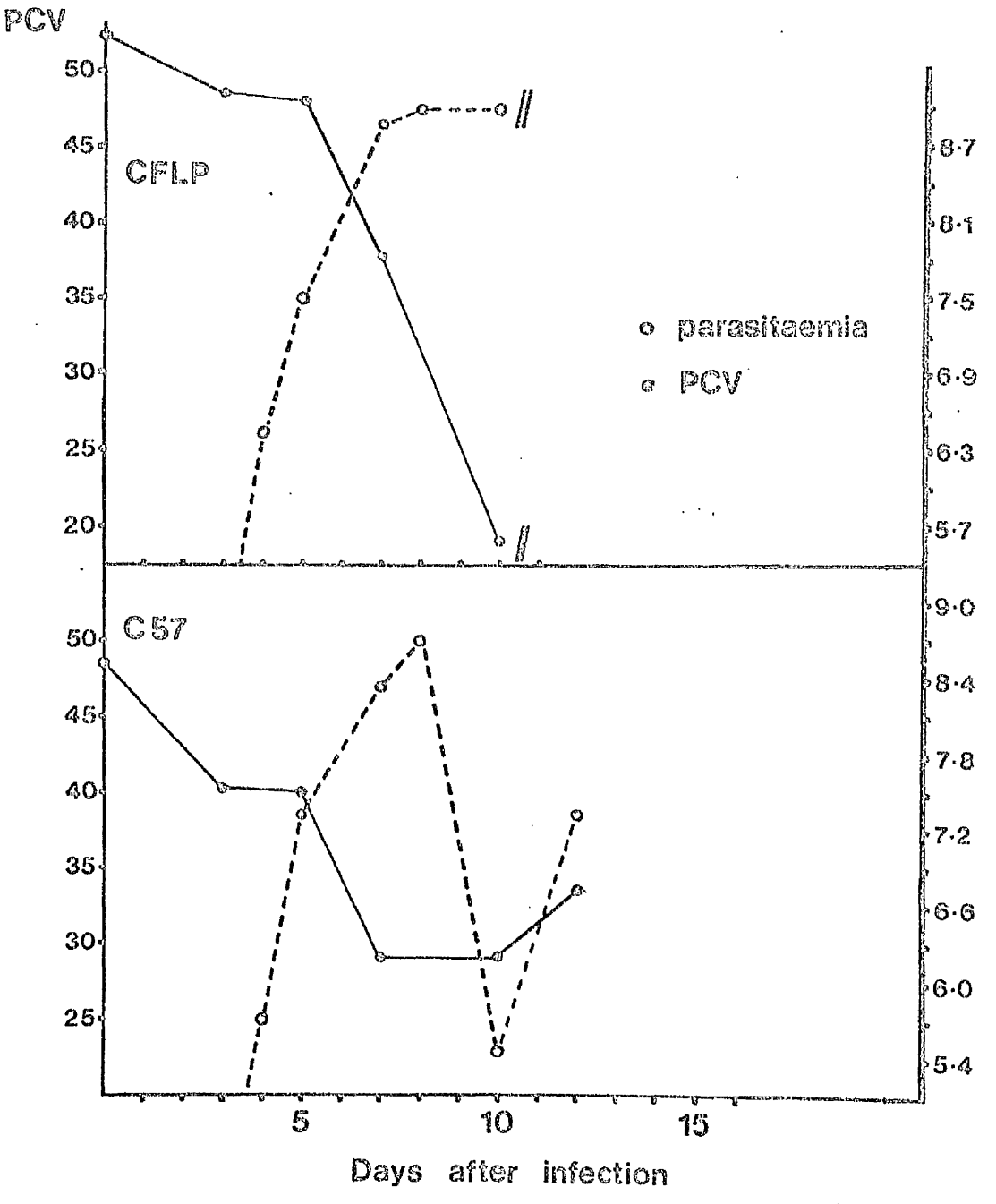
These initial experiments confirmed and extended the previous observation of the tolerance of C57Bl mice (Ssenyonga, 1974). All subsequent experiments were done with T. congolense GVR 1, using CFLP and C57Bl/6 mice as models of susceptible and trypanotolerant strains respectively to investigate possible mechanisms of resistance.

Pattern and course of T. congolense infection in C57Bl and CFLP mice. Groups of C57Bl and CFLP mice (30 of each strain) were infected with T. congolense GVR 1. Ten of each group were monitored daily for PCV and parasitaemia, and the remainder monitored for survival. The results presented in Fig 3.1 show that there is a similar development of parasitaemia and anaemia in both strains during the first 5 days after infection. By 7 to 8 days, however, the C57Bl are able to control and reduce the peripheral parasitaemia and

Table 3.3 Effect of genotype on the susceptibility of C57Bl mice

Mouse strain	No. of mice	Mean survival time (Days)
C57Bl/6	15	84 ± 8.1
C57 Bl/TB	12	91 ± 7.6
C57Bl/Bl0.D2	10	95 ± 4.3
C57Bl/10 Sc.Sn	10	77 ± 13.5
CFLP	8	8.7 ± 0.8

Fig 3.1 The development of anaemia and parasitaemia in C57Bl and CFLP mice after infection with *T. congolense* GVR 1.


PCV
50
45
40
35
30
25
20
CFLP
C57
parasitaemia
PCV
8·7
8·1
7·5
6·9
6·3
5·7
9·0
8·4
7·8
7·2
6·6
6·0
5·4
5
10
15
Days after infection

arrest the drop in PCV. CFLP mice, on the other hand, fail to reduce the high peripheral parasitaemia, suffer a progressive anaemia and die by day 10. In addition, the peak parasitaemia is slightly lower in C57Bl mice and although the initial increase in peripheral parasitaemia is similar in the two strains, from day 5 onwards the rate of growth is apparently slower in C57Bl mice. The infection progresses in C57Bl mice and displays the typical remissions and peaks of parasitaemia found in trypanosome infection (Fig 3.2). With passing time, the general trend is towards an overall decrease in peripheral parasitaemia with very gradual recovery of PCV. The survival profile of both strains is shown in Fig 3.3.

Red cell survival. Examination of the ^{51}Cr red cell survival curves (Fig 3.4) showed that although there was a shortened survival of labelled cells between day 6 and day 10 in parasitized CFLP mice, these mice died before this difference became statistically significant from controls. In C57Bl mice the initial disappearance rate was the same in both control and infected mice, but from day 10 onwards the half-life in the infected animals was dramatically reduced (Table 3.4) and continued throughout the chronic infection. (Fig 3.4c and unpublished observations).

Plasma and red cell volumes. The results of these estimations show that plasma volumes in mice of both strains were elevated by the 8th day of infection and that the circulating red cell volumes were lower than the corresponding control values (Fig 3.5). Since

Fig 3.2 Packed cell volume (PCV) and parasitaemia in C57Bl mice infected with *T. congolense* GVR 1.

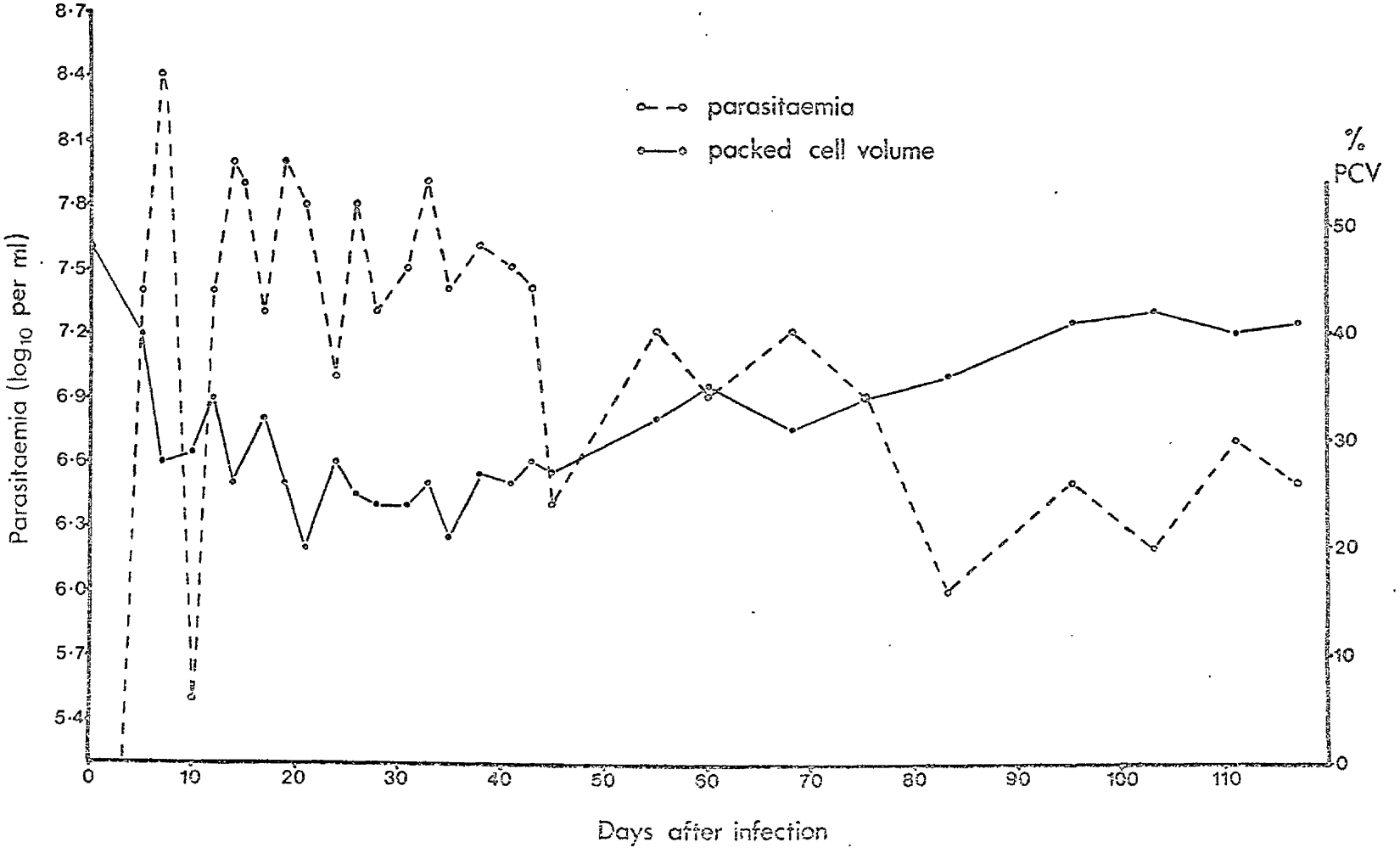

parasitaemia
packed cell volume
Parasitaemia (log_{10} per ml)
%
PCV
8·7
8·4
8·1
7·8
7·5
7·2
6·9
6·6
6·3
6·0
5·7
5·4
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
Days after infection

Fig 3.3 Survival of C57Bl and CFLP mice after infection with *T. congolense* GVR 1.

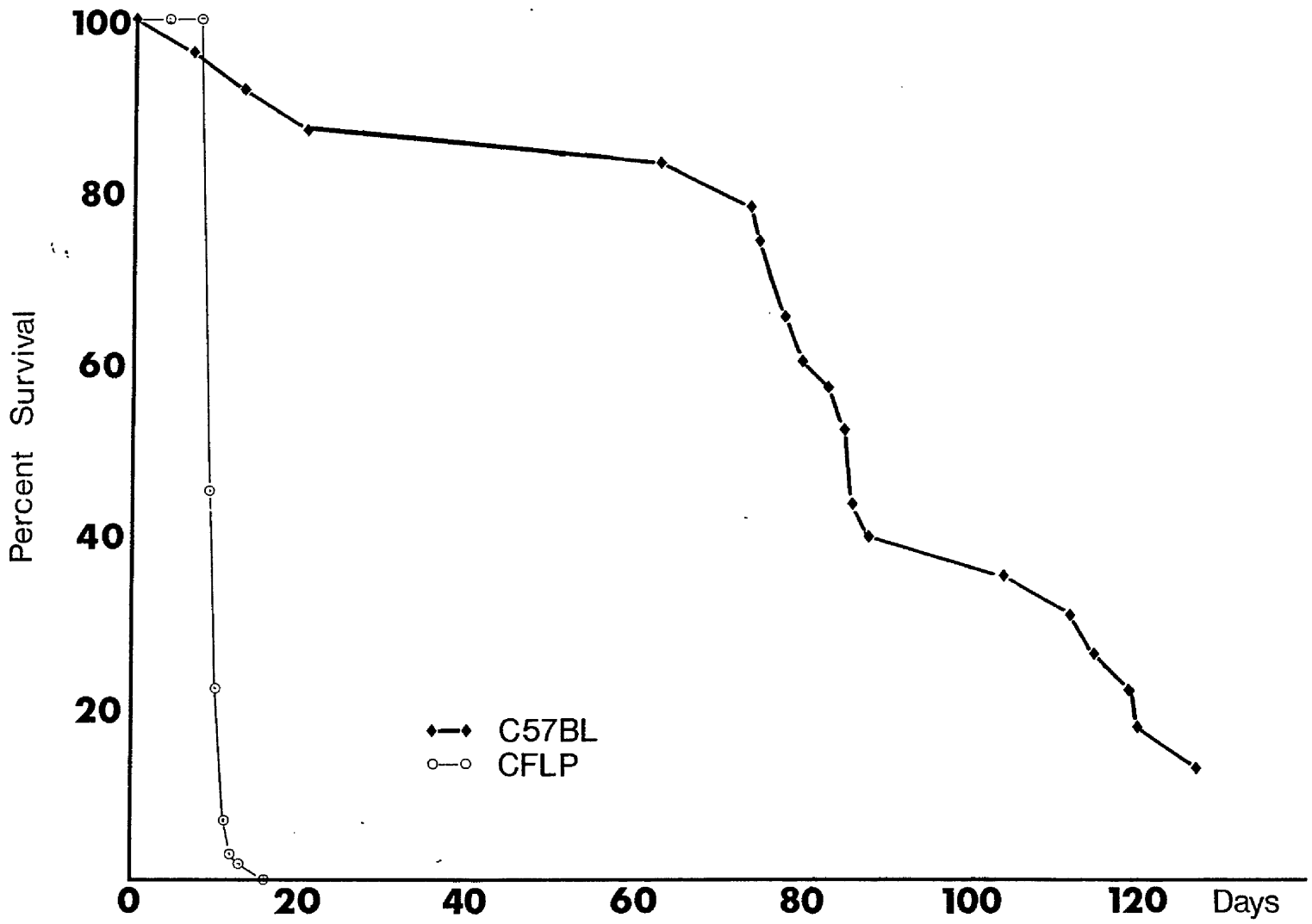

100
80
60
40
20
Percent Survival
C57BL
CFLP
0
20
40
60
80
100
120
Days

Table 3.4

	Day	Intercept a-$\log_{10}$	Slope	Correlation coefficient	Half-life ($T_{\frac{1}{2}}$) days
a) Control	0-10	104.4 ± 2.06	0.023 ± 0.0016	0.9713	13.2 ± 0.93
Infected	0-6	101.0 ± 1.39	0.021 ± 0.0018	0.9959	14.4 ± 1.02
	6-10	139.9 ± 15.86	0.046 ± 0.0046	0.9999	6.7 ± 0.75
b) Control	0-20	100.5 ± 3.64	0.021 ± 0.0009	0.9963	14.2 ± 0.57
Infected	0-10	101.5 ± 2.63	0.019 ± 0.0014	0.9950	15.9 ± 1.26
	13-20	1464 ± 542.6	0.122 ± 0.0074	0.9903	3.08 ± 0.22
c) Control	0-10	100.5 ± 3.64	0.021 ± 0.0009	0.9963	14.2 ± 0.57
Infected	0-10	97.9 ± 6.41	0.125 ± 0.0149	0.9980	2.7 ± 0.37

Labelled cells were injected into a) CFLP and b) C57 Bl mice on the day of infection and into c) C57 Bl mice 9 weeks after infection.

Table 3.5

	Units	Control CFLP	8-day CFLP	P	Control C57	8-day C57	P
Lactate dehydrogenase	IU	673±52.0	4810±733	0.001	685±49	4567±196	0.001
Aspartate transaminase	IU	78.8±12.8	512.2±121.5	0.01	102.3±7.7	253±9.3	0.001
Alanine Transaminase	IU	27.4±2.5	285.8±97.4	0.05	41.3±3.5	73.3±6.3	0.02
Glucose	mmol/l.	9.35±0.34	5.83±0.82 1.25±0.05**	0.01 0.001	9.0±0.7	6.72±0.66	N.S.
Albumin	g/l	17.0±1.9	11.4±1.1	0.05	19.6±0.3	12.8±1.1	0.01
Total globulin	g/l	33.4±1.36	35.6±1.39	N.S.	26.1±1.73	32.2±0.85	0.02
Total protein	g/l	50.4±1.5	47.0±0.8	N.S.	45.7±1.8	45.0±0.7	N.S.
Albumin/Globulin	-	0.5±0.07	0.34±0.05	N.S.	0.77±0.07	0.4±0.04	0.01
Urea	mmol/l	7.04±1.08	8.66±0.65 26.45±1.95	N.S. 0.001	7.48±0.26	8.6±0.68	N.S.
Phosphate	mmol/l	3.74±0.14	4.64±0.16 7.42±0.88	0.01 0.02	3.39±0.18	3.58±0.13	N.S.
IgG_1	mg/ml	2.39	2.18±0.07*	-	2.47	2.42±0.08*	-
IgG_2	mg/ml	1.03	1.16±0.07*	-	1.17	1.99±0.09*	-
IgM	mg/ml	0.23	0.98±0.04*	-	0.23	1.48±0.10*	-

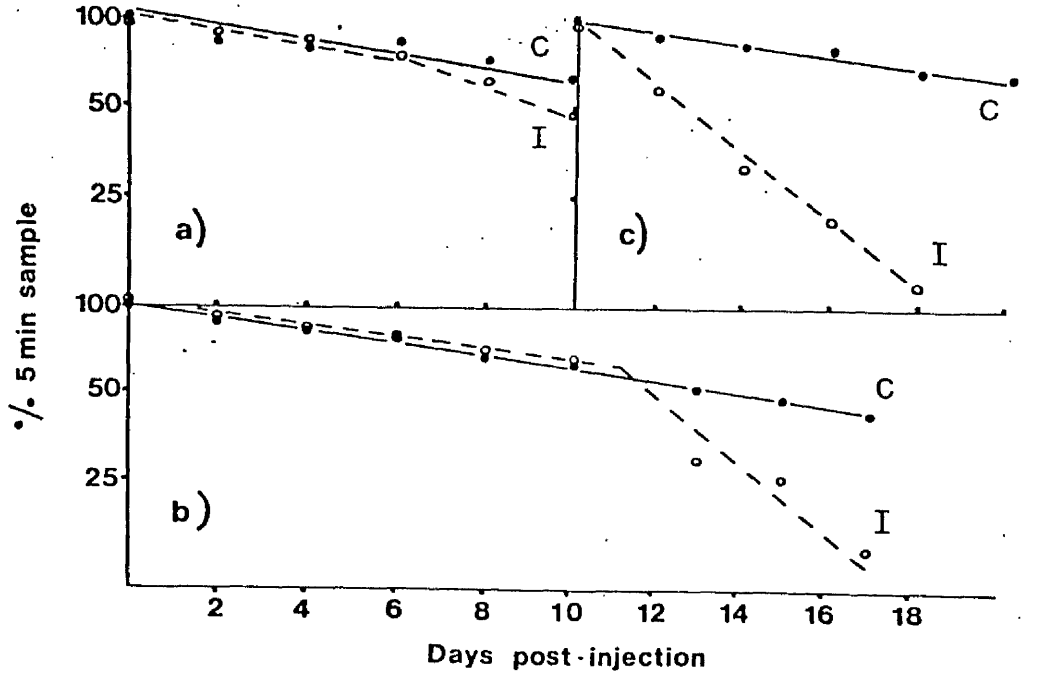
*Estimated on day 10. Control values were measured on pooled batch samples. **Estimations performed on moribund mice. Only 2 per group. No significant alterations occurred to the following: creatinine, bilirubin, alkaline phosphatase, electrolytes and creatinine phosphokinase.

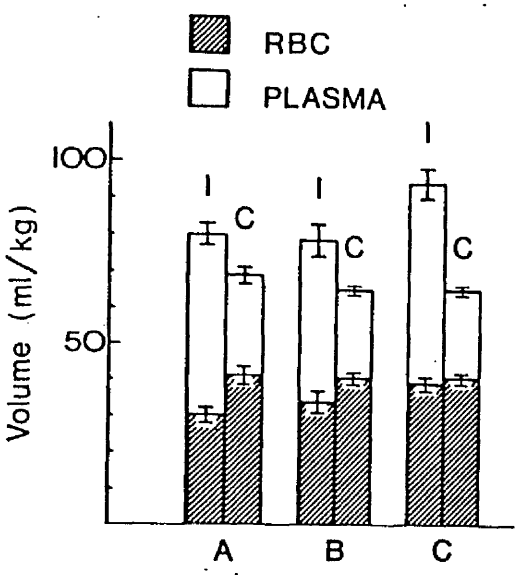
Fig. 3.4. ^{51}Cr red cell disappearance curves in CFLP and C57Bl mice: labelled cells were injected into a) CFLP and b) C57Bl mice on the day of infection and into c) C57Bl mice 9 weeks after infection.

C = control, I = infected.

Fig. 3.5. Plasma and red cell volumes of mice infected with *T. congolense* GVR 1: group A - CFLP mice, group B and C - C57Bl mice.

I - infected, C - control. Plasma volumes were measured on the 8th day of infection in groups A and B, and after a 9-week infection in group C.





PROBABILITY (P)			
V_{rbc}	< 0·01	< 0·05	NS
V_{plasma}	< 0·001	< 0·001	< 0·001
$V_{whole\ blood}$	< 0·05	< 0·05	< 0·001

in both strains there was an increase in the total blood volume, it is apparent that the anaemia which develops in both strains by the 8th day of infection is essentially caused by a combination of haemodilution and accelerating red cell loss.

In the chronic infection of C57Bl mice the plasma volumes were markedly elevated, but circulating red cell volumes were comparable to control values despite a greatly decreased red cell survival rate. This suggests that erythropoiesis must be greatly increased in such animals and that the depressed haematocrits are largely brought about by haemodilution.

Blood biochemical determinations. By the eighth day of infection striking and similar changes were evident in the biochemical indices of both strains (Table 3.5). Lactate dehydrogenase, aspartate transaminase and alanine transaminase were markedly increased although whether the increased transaminase activities were due to specific organ damage or derived from the parasites is not known (Moon, Williams and Witherspoon, 1968). Glucose and albumin levels were decreased; as a consequence of the latter the albumin/globulin ratios in both strains were reduced by the eighth day, although in C57Bl mice the total protein levels were unaltered due to an increase in globulin. Electrolytes and other blood indices remained essentially unchanged, or were equally altered in both strains. In addition, in CFLP mice, moribund prior to death between 8 and 10 days post-infection, glucose levels were greatly reduced, urea levels increased four-fold and serum phosphate two-fold.

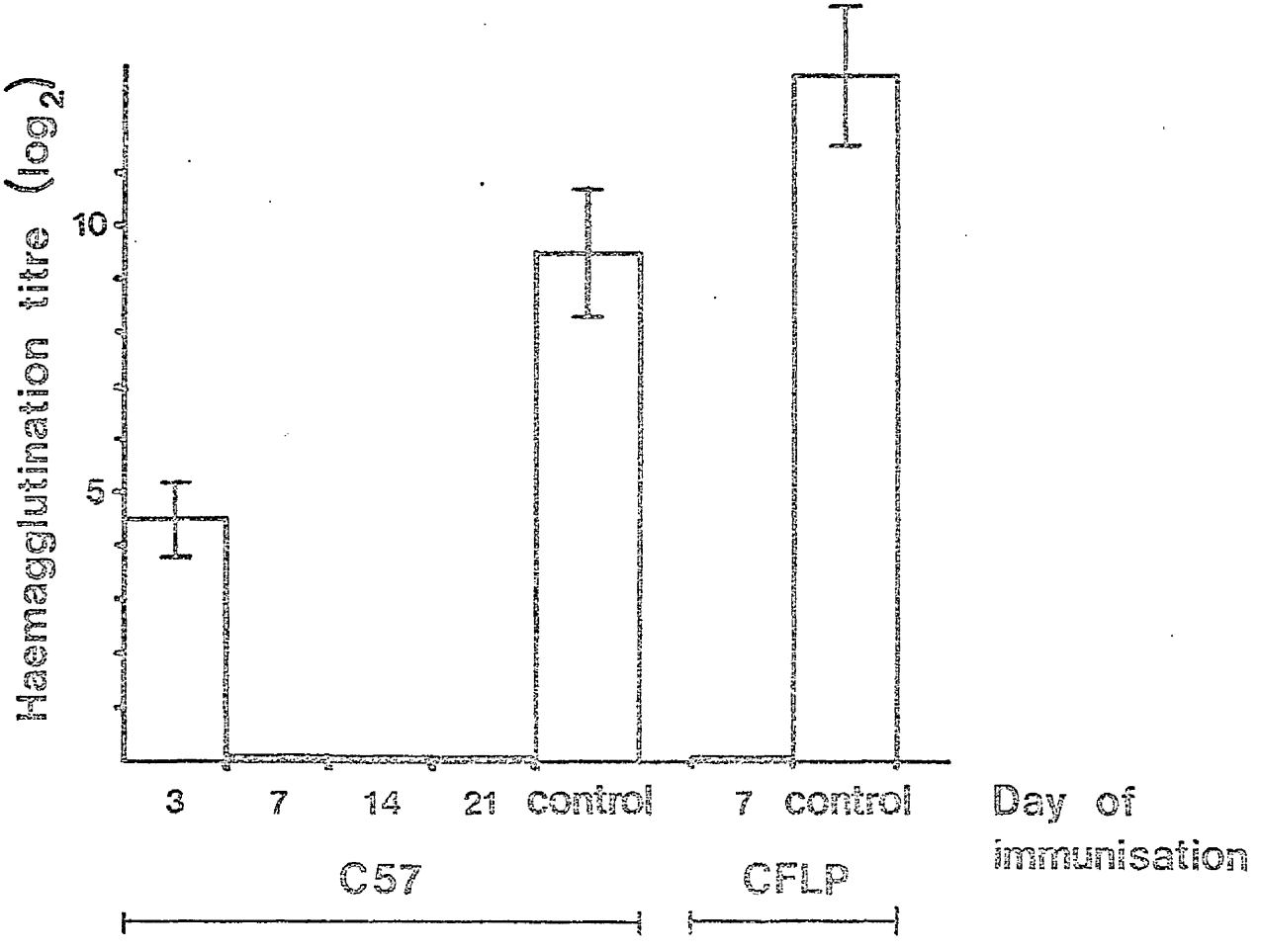
In chronic infections of C57Bl mice all of these indices returned to near normal , with the exception of albumin, which remained slightly depressed and globulin and lactate dehydrogenase which remained elevated.

Immunoglobulin levels. Levels of IgG_1 were essentially unchanged but by the tenth day of infection IgM levels were elevated in both strains, the increase being more pronounced in C57Bl mice. At this time IgG_2 levels were also increased in the C57Bl mice but remained unaltered in the CFLP mice (Table 3.5).

Humoral response to horse red blood cells. Haemagglutinating antibody responses to HRBC were totally suppressed in both strains of mice if antigen was administered after 7 days post-infection (Fig 3.6), and they remained suppressed in the surviving C57Bl mice.

In the above experiments, no major difference was found in the two strains of mice in the degree of physiological aberration, as a consequence of trypanosome infection, which would account for the reduced susceptibility of C57Bl mice and their ability to survive an infection which kills CFLP mice on the 8th to 10th day. Consequently, further experiments were done on the basis that differences in susceptibility were a reflection of differences in the immune response to the trypanosome.

Fig 3.6. Haemagglutination titres against horse red blood cells in mice infected with T. congolense GVR 1.


Haemagglutination titre (log_2)
10
5
3
7
14
21
control
7
control
Day of immunisation
C57
CFLP

The course of T. congolense infection in the absence of antibody. To investigate the role of the immune response in the control of parasitaemia in C57Bl mice, 2 groups of C57Bl mice were immunosuppressed either by total body irradiation (650 rad) the day before infection, or with a single dose (300 mg/kg) of cyclophosphamide 3 days before infection. The response of these mice was compared to a control group by monitoring tail blood for parasites. Fig 3.7 shows the result of cyclophosphamide pretreatment, the effect of which was exactly the same as total body irradiation (result not shown). As a consequence of either treatment, mice are unable to reduce and control the initial parasitaemia, thus suggesting that the ability of C57Bl mice to control and survive infection is influenced by the presence of an intact immune response.

Clearance of ^{75}Se-labelled T. congolense by infected C57Bl and CFLP mice. The use of ^{75}Se-labelled T. brucei in assessing the immune response in mice is the subject of chapter 4, and the technique works equally well with T. congolense. Consequently, ^{75}Se-labelled T. congolense were used to compare the immune response of CFLP and C57Bl mice which had been infected 9 days previously.

C57Bl mice have a higher hepatic uptake than both uninfected mice of the same strain and infected CFLP mice (Table 3.6). The higher uptake of the latter over its uninfected counterpart can be explained by the increase in liver weight as a consequence of infection, but the same cannot account for the marked elevation in infected C57Bl mice.

Fig 3.7. Parasitaemic profiles and survival of C57Bl mice infected with T. congolense following treatment 3 days previously with cyclophosphamide compared with untreated controls.

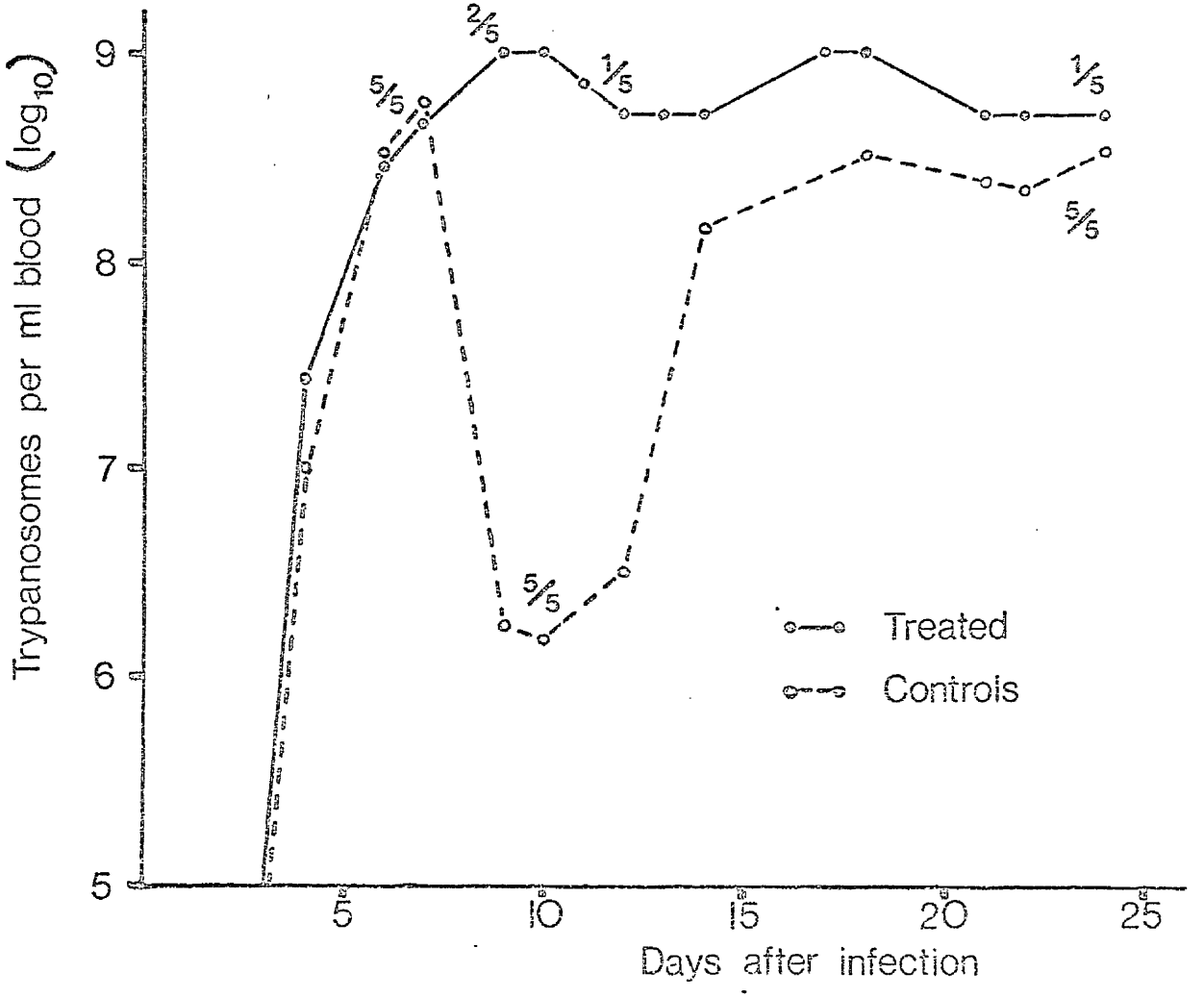

Trypanosomes per ml blood (log_{10})
9
8
7
6
5
5
10
15
20
25
Days after infection
2/5
5/5
1/5
1/5
5/5
5/5
Treated
Controls

Table 3.6 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 3.7 Tissue distribution of ^{75}Se-labelled T. congolense by infected CFLP mice and following chemotherapy

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

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

Furthermore, the inability of CFLP mice to remove radiolabelled parasites from the circulation is overcome by prior chemotherapy. Eight days after infection with T. congolense GVR 1, a group of CFLP mice were treated with Berenil and injected 24 hr later with radiolabelled trypanosomes. The results (Table 3.7) show that chemotherapy increases the ability of CFLP 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%.

The effect of splenectomy on survival of C57Bl mice. The importance of an intact immune system on the outcome of infection in C57Bl mice was further illustrated in animals which had undergone surgical removal of the spleen two weeks prior to infection. 80% mortality occurred in such mice over the first 3 weeks compared with no deaths in the intact group (Fig 3.8). The involvement of antibody in the inability of splenectomised C57Bl mice to overcome the effect of infection was shown in 3 ways:

a) Infectivity neutralisation test. The tests conducted with sera collected 10 days post-infection from the 2 strains showed that trypanocidal activity was present only in serum collected from C57Bl mice, and prior splenectomy removed this capability (Table 3.8).

b) A hepatic uptake assay, similar to those described above, was done on intact and splenectomised C57Bl and CFLP mice on the ninth day after infection with T. congolense GVR 1. The results (Fig 3.9)

Fig. 3.8 Survival of C57Bl mice after surgical removal of the spleen 2 weeks prior to infection with *T. congolense* GVR 1.

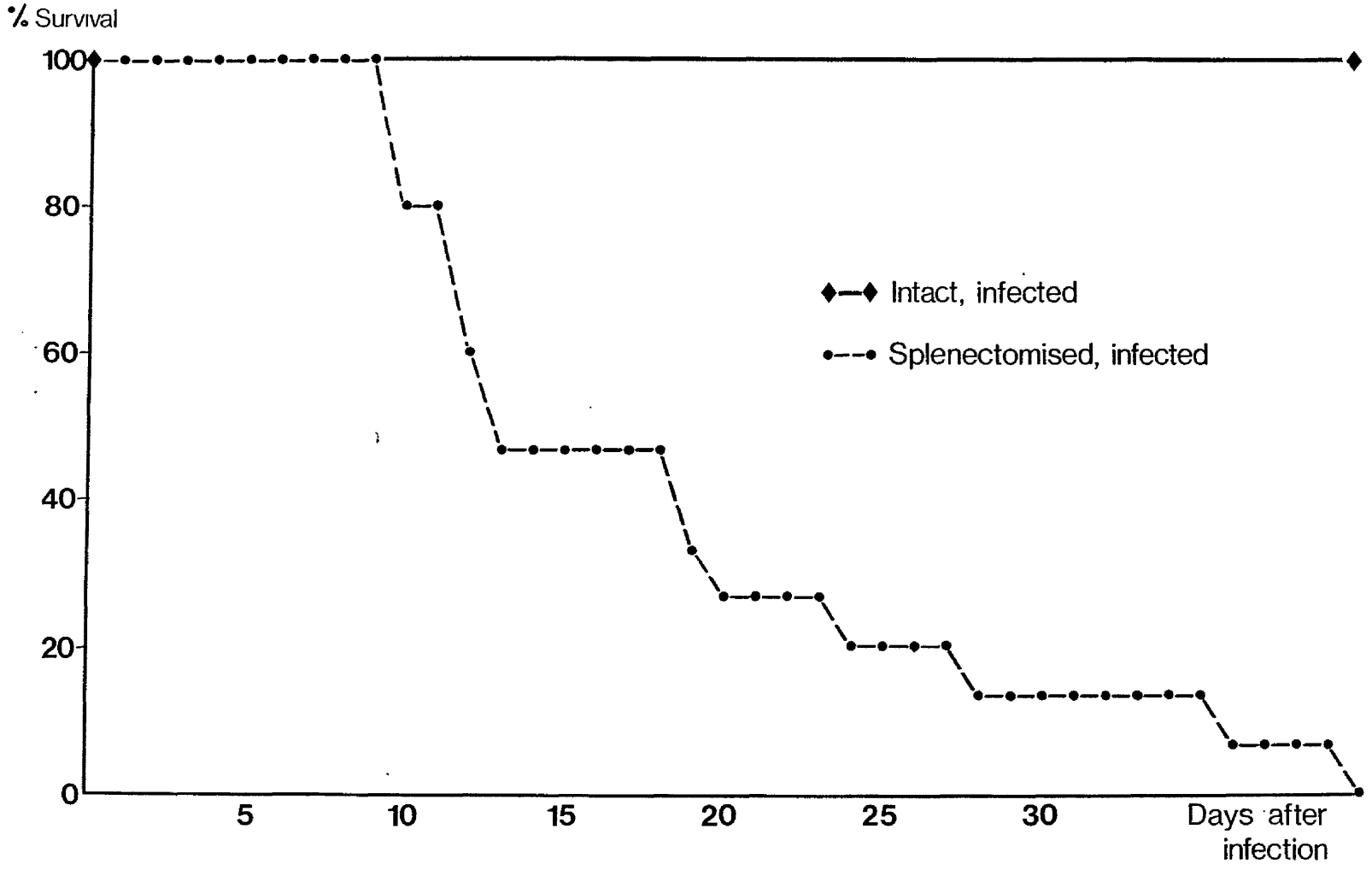

% Survival
100
80
60
40
20
0
5
10
15
20
25
30
Days after infection
Intact, infected
Splenectomised, infected

Table 3.8 Infectivity neutralisation test on sera from C57Bl and CFLP mice infected with *T. congolense* GVR 1

Day 10 serum from :	Survival of susceptible recipient mice
Splenectomised CFLP	0/12
Intact CFLP	1/12
Splenectomised C57Bl	0/11
Intact C57Bl	9/13
Normal serum from CFLP	0/6
Normal serum from C57Bl	0/6

Table 3.9 Trypanolysis of ^{75}Se-labelled *T. congolense*

Group	% lysis
Splenectomised CFLP*	21.1 ± 1.6
Intact CFLP	25.5 ± 2.3
Splenectomised C57Bl	22.4 ± 1.2
Intact C57Bl	58.1 ± 1.8
Normal mouse serum	18.4 ± 2.0
PSG	15.0 ± 1.8
MP40	85.2 ± 1.4

*Pooled serum from mice killed on days 9 and 10 of infection

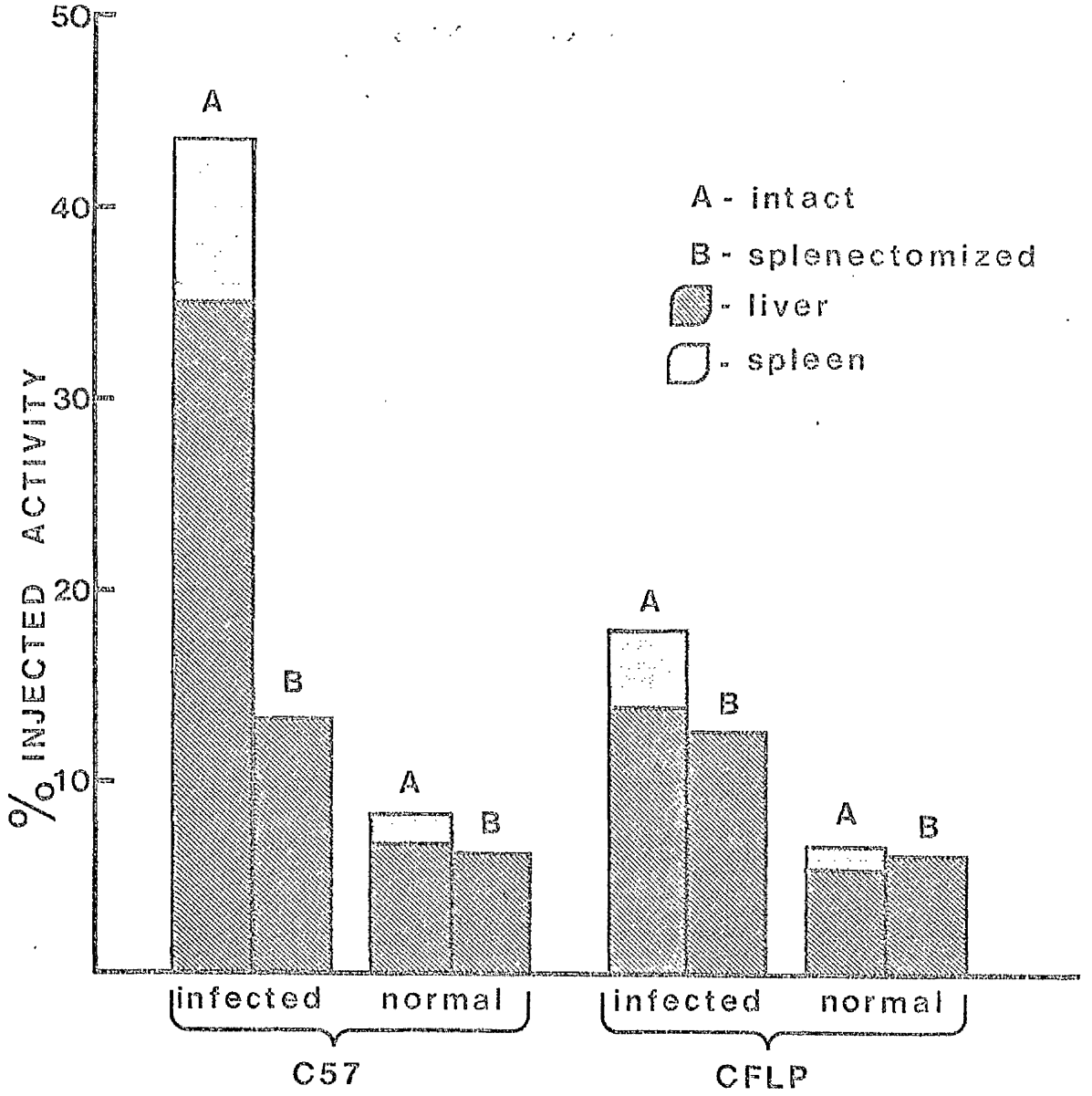
show that only in the case of intact C57Bl mice was there the capability to remove radiolabelled parasites from the circulation.

c) ^{75}Se-labelled *T. congolense* trypanolysis. The superior immune response of intact C57Bl mice was evident in a trypanolysis assay in which ^{75}Se-labelled trypanosomes were incubated with day 10 sera from infected mice, in the presence of guinea pig serum as a source of complement. The release of ^{75}Se-activity into the supernatant was used as a measure of the trypanolytic ability of the respective sera. The results (Table 3.9) confirmed the findings of the infectivity neutralisation and hepatic uptake assays. Marked trypanolytic activity was present only in the serum from intact C57Bl mice.

Thus, the ability of C57Bl mice to survive infection while CFLP mice succumb presumably reflects the difference in the effectiveness of the immune response, either in quantity or quality of antibody formation, or the rate at which antibody is formed. Presumably, the ability to maintain effective levels of antibody, possibly IgM (*vide supra*), accounts for the capability of the C57Bl strain, and consequently experiments were designed which attempted to enhance the immune response of CFLP mice, thus decreasing their susceptibility to *T. congolense* infection.

Passive immunisation of CFLP mice. The above experiments consistently indicated, by a variety of methods, that serum from

Fig. 3.9. Hepatic and splenic uptake of T. congolense labelled with ^{75}Selenomethionine in intact and splenectomised C57B1 and CFLP mice. The assay was performed on the 9th day after infection.


50
40
30
20
10
% INJECTED ACTIVITY
A
B
A
B
A
B
A
B
A - intact
B - splenectomized
- liver
- spleen
infected
normal
infected
normal
C57
CFLP

infected C57Bl mice is highly active against trypanosomes. Consequently, serum from C57Bl mice was injected into CFLP mice, following a range of regimens, in an attempt to confer protection against T. congolense GVR 1 infection. The results are shown in Table 3. 10. Serum collected at various times from C57Bl mice, and injected into CFLP mice at various stages of infection, made no appreciable differences in survival, all recipients dying by day 12 of infection. Similarly, normal C57Bl was completely ineffective.

Passive immunisation was also attempted using hyperimmune serum from CFLP mice. This had been raised in CFLP mice by Berenil chemotherapy of a 7 day T. congolense infection, followed by two challenges on days 7 and 21 days after treatment, to which the mice were resistant. The hyperimmune serum (HIS) was collected 5 days after the second challenge. These mice were assumed to be immune because they resisted challenge, but confirmation was sought in an infectivity neutralisation test using 1/5 and 1/10 dilutions of the serum. These doses were selected to eliminate the possibility of the serum being diluted to below protective levels when subsequently injected into susceptible hosts. At both dilutions, 100% protection against infection was afforded to recipient mice. The possibility of residual Berenil influencing the viability of the challenge inocula was discounted, since under these conditions Berenil is not prophylactic against T. congolense (see Chapter 2). HIS was unable to produce protection in mice which received it, and even when given at the time of infection produced only partial protection, as indicated by a delay in patency (Table 3.10).

Table 3.10 Attempted passive immunisation of CFLP mice infected with T. congolense GVR 1, using serum from infected C57Bl mice, hyperimmunised CFLP mice and normal C57Bl mice.

Donor mice	Day of collection of donor serum after infection	Day of infection of recipient CFLP mice on which they received donor serum	Amount injected (i.p.) ml/day	Survival of recipient (at day 12 post-infection)
Infected C57Bl	10	5 + 6 + 7 + 8	0.2 ml	0/5
	10	5	0.5 ml	0/5
	10	9	0.5 ml	0/5
	1, 3, 5, 7	1 + 3 + 5 + 7	0.2 ml	0/6
	6, 7, 8, 9	6 + 7 + 8 + 9	0.2 ml	0/6
Hyperimmune CFLP	not applicable	-1 + 0 + 1	0.2 ml	0/5 at day 15*
	" "	5 + 6 + 7	0.2 ml	0/5
	" "	8 + 9	0.5 ml	0/7
Normal C57Bl	" "	1 + 3 + 5 + 7	0.2 ml	0/6
	" "	6 + 7 + 8 + 9	0.2 ml	0/6

*All mice died, but showed an extended prepatent period.

Effect on subsequent parasitaemia of different infection dose.

To investigate if CFLP mice require a longer time to mount an effective immune response to control the parasitaemia, time which they may not be allowed with an inoculum of 10^4 organisms, groups of mice were infected with 10^1, 10^2, 10^3 and 10^4 T. congolense GVR 1, and monitored for the appearance of parasites. As might be expected, a reduction in infective dose was accompanied by a lengthening of the prepatent period (Fig 3.10). However, despite an indication that with lower dose the time from detection of parasites to death was slightly longer, in none of the groups was initial parasitaemia controlled, and all mice died with fulminating parasitaemias.

Effect of simultaneous inoculation with irradiated trypanosomes.

An attempt was made to enhance the antigenic priming of CFLP mice by inoculating a dose of 1×10^7 trypanosomes rendered incapable of replication by gamma irradiation at the time of infection with 5×10^2 normal trypanosomes. The results (Fig 3.11) show that mice given such a combination of trypanosomes had a prolonged prepatent period but, following patency, the parasitaemic profile was essentially similar to that of mice which received only normal infective trypanosomes. Those mice given only irradiated trypanosomes failed to develop patent infections.

Immunopotentiation of the mononuclear phagocytic system (MPS)

The basis of this approach is that various bacterial and chemical compounds cause hypertrophy and hyperplasia of the MPS. Some have

Fig 3.10. Parasitaemic profiles of CFLP mice infected with different doses (10^1, 10^2, 10^3, 10^4) of T. congolense GVR 1.

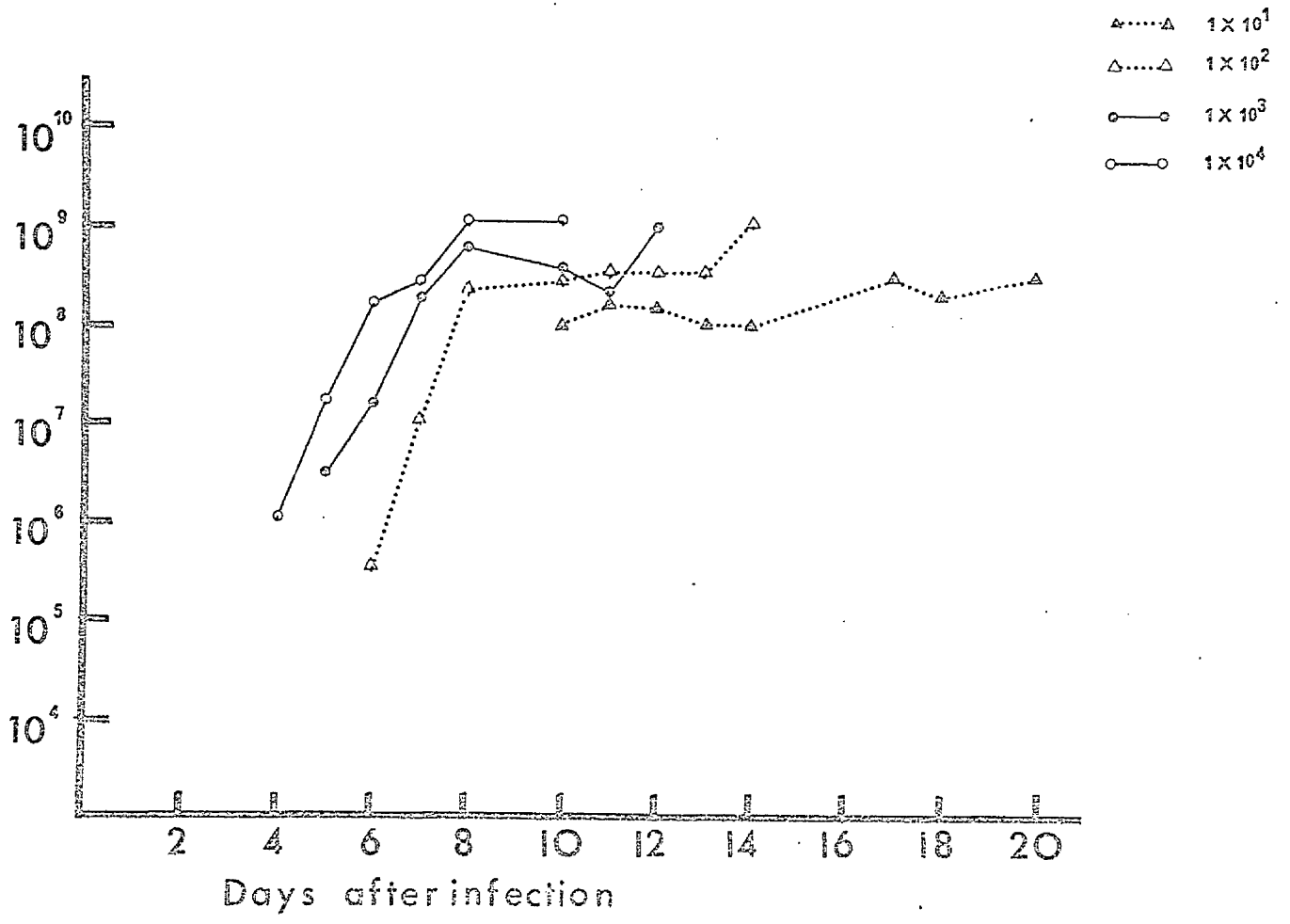
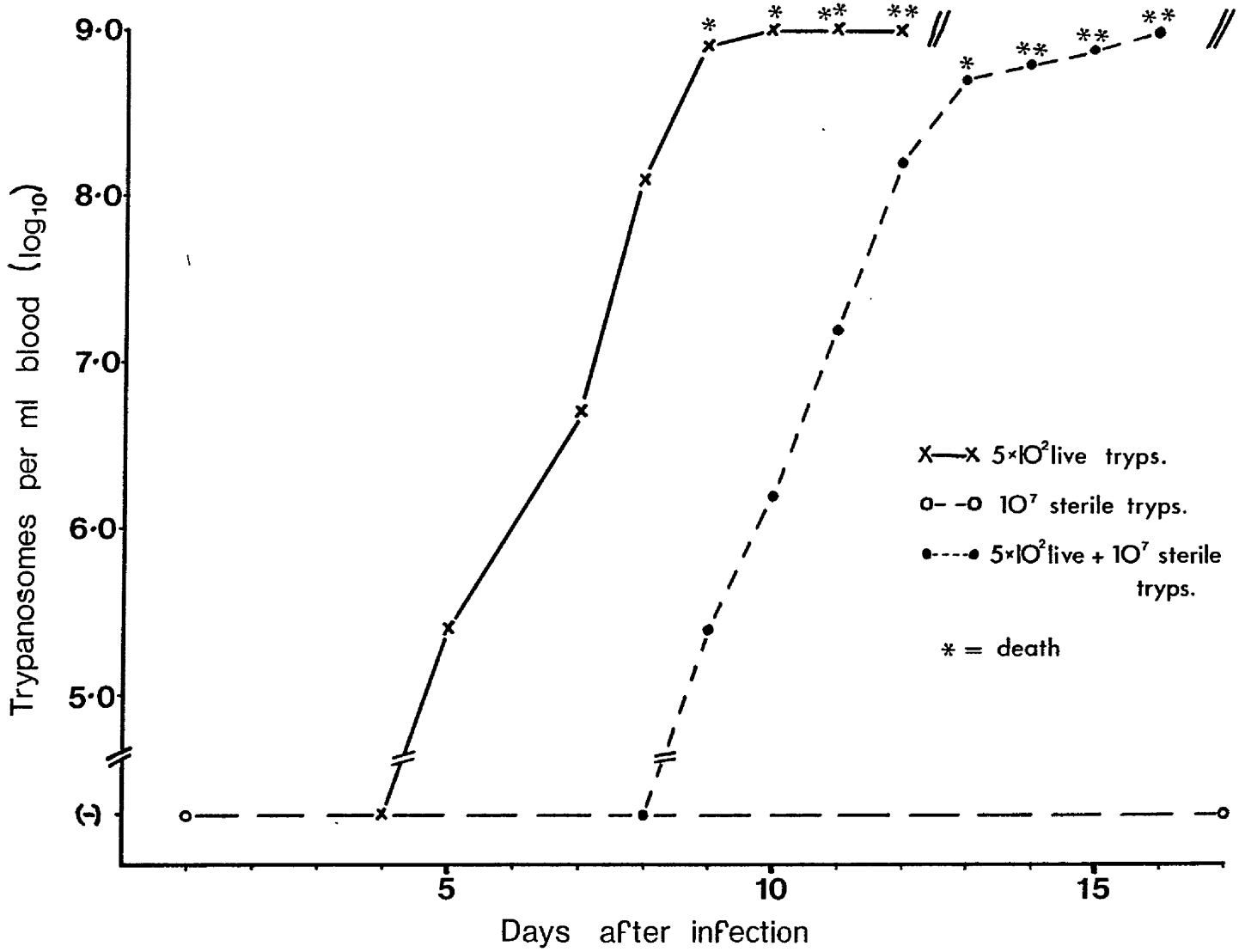

1×10^1
1×10^2
1×10^3
1×10^4
10^{10}
10^9
10^8
10^7
10^6
10^5
10^4
2
4
6
8
10
12
14
16
18
20
Days after infection

Fig. 3.11. Parasitaemic profiles and survival of CFLP mice inoculated with viable and/or non-replicative *T. congolense* GVR 1.

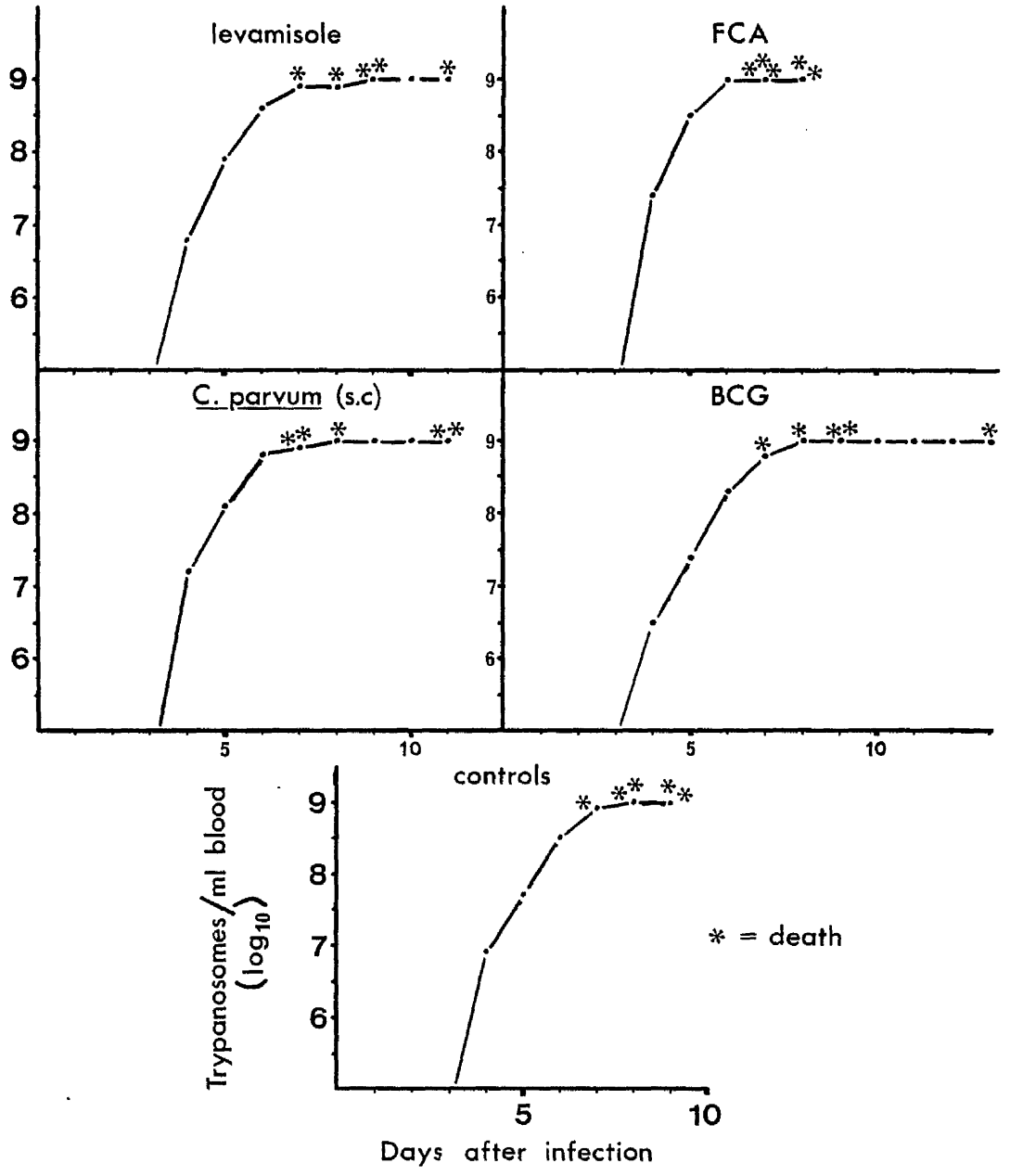

9·0
8·0
7·0
6·0
5·0
(-)
Trypanosomes per ml blood (log_{10})
5
10
15
Days after infection
X—X 5×10² live tryps.
o- -o 10⁷ sterile tryps.
•----• 5×10² live + 10⁷ sterile tryps.
* = death

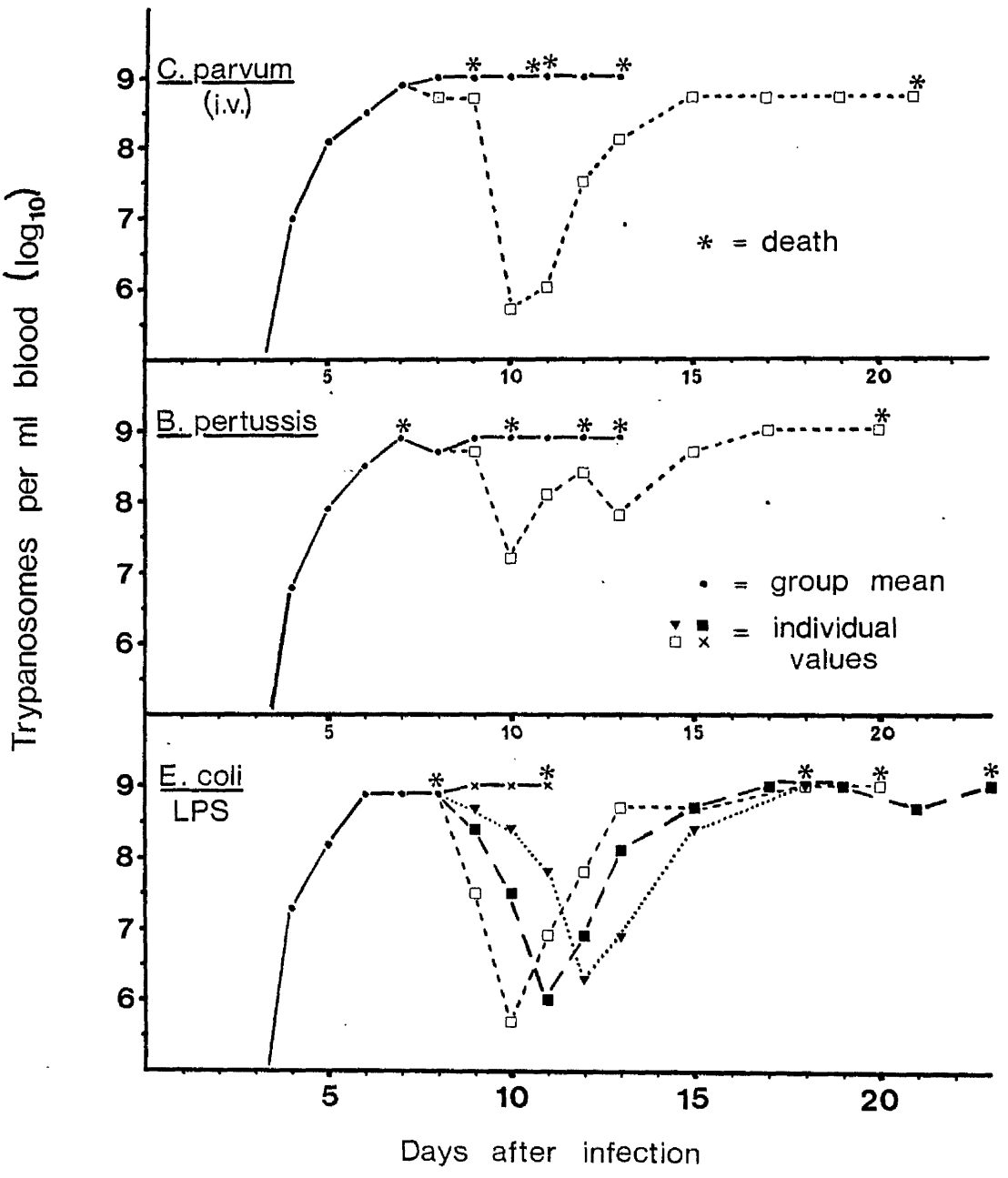
been reported to confer a degree of non-specific immunity against protozoan infections (Nussenzweig, 1967; Clark, Allison and Cox, 1976; Clark, Cox and Allison, 1977), thus the injection of various substances was carried out in an endeavour to enhance the activity of the MPS and thereby facilitate a reduction in the peripheral parasitaemia.

The results are presented in Fig 3.12. Neither levamisole, CFA, C. parvum (s.c.) nor any of the regimens using BCG produced any prolongation of the infection in CFLP mice. However, with C. parvum (i.v.), B. pertussis and E. coli there was some evidence of a regulatory effect on the infection, although some animals in each group died within the normal period. Thus, with both C. parvum (i.v.) and B. pertussis, one mouse from each group survived the first peak by successfully modulating the parasitaemia, only to die during the second peak. A similar outcome was observed in 3 mice treated with E. coli LPS, none surviving beyond 23 days. In conclusion, therefore, hyperplasia and hypertrophy of the MPS alone does not confer any marked degree of protection in the absence of an effective antibody response.

Fig. 3.12. Parasitaemic profiles and survival of CFLP mice following treatment with immunostimulants.

The result presented for BCG pretreatment is representative of that achieved with all of the treatment schedules described in the text.


levamisole
FCA
C. parvum (s.c)
BCG
controls
Trypanosomes/ml blood (log_{10})
9
8
7
6
5
10
* = death
Days after infection


C. parvum
(i.v.)
* = death
B. pertussis
• = group mean
= individual values
E. coli
LPS
Trypanosomes per ml blood (log10)
Days after infection
9
8
7
6
5
10
15
20

DISCUSSION

Field observations in Africa have long since indicated that certain species of animal are able to live in tsetse areas in which it is impossible for other species to exist, since they rapidly succumb to trypanosomiasis. Thus the concept of trypanotolerance was realised, and it has been shown to occur in cattle (Stewart, 1951; Desowitz, 1959; Roberts and Gray, 1972; Toure, 1977; Murray, Morrison, Murray, Clifford and Trail , 1979), in sheep and goats (Toure, 1977; Griffin and Allonby, 1979) and in certain species of wildlife (Ashcroft, Burtt and Fairbairn, 1959).

The demonstration that certain inbred strains of mice possess an inherent increased resistance to trypanosome infection (Ssenyonga, 1974) made it possible to conduct experiments to investigate the mechanisms of trypanotolerance in a host with a defined genetic background, such studies being very difficult in domestic livestock through their genetic heterogeneity.

The findings presented in this chapter confirmed the superior resistance of the C57Bl strain to T. congolense infection, but showed that the resistance was related to the virulence of the parasite. Thus, infection with T. congolense LUMP 52 and LR 1 results in a rapidly fulminating parasitaemia and death within 10 days in all strains of mice. Such an influence has been reported with T. brucei infections in mice (Clayton, 1978; Sacks, Selkirk, Ogilvie and Askonas, 1980), and indicates that

susceptibility may be influenced by a variety of factors. With the parasite selected for the remainder of the study, T. congolense GVR 1, significant resistance was shown only by C57Bl mice, and there was no difference in susceptibility of different sub-strains of this line. It has also been shown that three resistant strains of C57Bl mice, congenic at the H-2 locus, have similar resistance to T. congolense infection, showing that susceptibility is not associated with the H-2 haplotype (Morrison, Roelants, Mayor-Withey and Murray, 1978).

It is interesting to note that despite the marked difference in susceptibility between C57Bl and CFLP mice to infection with T. congolense GVR 1, these 2 strains and indeed all strains of mice tested, responded remarkably similarly to T. brucei TREU 667. Why this should be was not investigated further, but may be associated with the additional extravascular component of T. brucei infection.

It was considered initially that the difference in mortality with T. congolense GVR 1 infection between C57Bl and CFLP mice might be associated with one or more of the following factors:- first, the degree of anaemia, secondly a difference in the pathophysiological changes associated with tissue damage and biochemical derangement: thirdly, the immunosuppressive effects characteristic of trypanosomiasis may be more severe in susceptible strains of mice: finally, the degree and duration of the parasitaemia in the two strains may differ.

With regard to the anaemia, the fall in PCV during the first 8 days post-infection was similar in both strains. Subsequently, it became stabilized in the resistant strain but continued to decline in the CFLP mice to very low levels. While, therefore anaemia may be significant as a final cause of death, its presence is entirely a consequence of the level and duration of the parasitaemia (Holmes and Jennings, 1976). Furthermore, in both strains the pathogenesis of the anaemia at this stage of the infection was similar, ie an accelerating rate of red cell destruction complicated by haemodilution, a situation similar to that reported in infected cattle (Holmes and Mamo, 1975).

Although biochemical analysis of the blood showed gross alterations in the levels of some indices by the eighth day of infection, these were similar in both strains. The only exception was in the case of moribund CFLP mice which had elevated urea and serum phosphate levels and severe hypoglycaemia, all of which are known to be associated with high parasitaemias (Goodwin and Guy, 1973; Herbert, Mucklow and Lennox, 1975). Elevated serum aspartate transaminase (AST) and alanine transaminase (ALT) values are indicative of diseases involving myocardial, hepatic and muscle damage, and have been reported in T. evansi infections in camels (Boid, Mahmoud and Gray, 1979). Changes in AST levels have also been recorded with T. vivax (Gray, 1963) and T. congolense (Wellde, Lotzsch, Deindl, Sadun, Williams and Warui, 1974) infections in cattle, and rabbits infected with T. brucei (Goodwin and Guy, 1973), but comparative estimations have not been done in breeds of different

susceptibility. Thus, in the absence of gross differences between the 2 strains of mice reported here, the pathophysiological changes over the first 8 days of infection must be regarded as being similar.

C57Bl mice were shown to be able to control the initial parasitaemia, and there was an indication that the rate of growth of peripheral parasitaemia may be an important factor in determining the outcome of infection. Thus, in C57Bl mice the rate of growth in peripheral parasitaemia slows from day 5 onwards, compared to an almost continual growth in CFLP mice, until parasite remission occurs in the resistant strain around day 8. In the meantime, death occurs in the susceptible CFLP strain. It has been suggested (Clarkson, 1980) that C57Bl mice modulate the virulence of the successive VATs which appear with passing time, and ultimately the bloodstream parasitaemia is represented by VATs of low pathogenicity, which accounts for the ability of the C57Bl strain to survive so long. However, this is not the case in the host/parasite system described here, since a population isolated from a C57Bl mouse on the 120th day of infection, when injected into CFLP mice, resulted in death within 10 days, associated with a fulminating parasitaemia (results not presented). Thus the virulence of the parasite was unaltered.

A major feature of both animal and human trypanosomiasis is hypergammaglobulinaemia, particularly IgM (Mattern, Masseyeff, Michel and Peretti, 1961; Luckins, 1972a), a large proportion of which would appear not to be specific for the trypanosome (Freeman, Smithers, Targett and Walker, 1970; Corsini, Clayton, Askonas and Ogilvie, 1977). At the same time the host becomes immunosuppressed.

The agglutination response of both strains to heterologous red cell antigen (Murray, Jennings, Murray and Urquhart, 1974a) was suppressed to the same degree in both strains by 7 days after infection which suggests that the degree of immunosuppression was not a contributary factor to death. It is known that chronically infected mice are able to limit relapsing parasitaemias and it has been suggested that this is achieved by a small residual production of anti-parasite IgM (Hudson and Terry, 1979; Selkirk and Sacks, 1980). A marked increase in IgM production occurs in C57Bl mice (Clarkson, 1976b), whereas there is virtually no increase in the highly susceptible C3H/mg strain. While the difference was not so marked in the system described here, CFLP mice did not produce as efficient a total IgM response as the resistant strain. These findings indicate that a more effective anti-trypanosome response may be produced in the resistant strain even when the response to HRBC and SRBC is suppressed. The nature of the antigen is important and the dependency upon T cell regulation, which is thought to govern the response to SRBC (Claman and Chaperon, 1969; Katz and Benacerraf, 1972), may influence the efficiency of the immune response during trypanosome infection, suggesting that trypanosome antigen elicits mainly an IgM response which 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), as judged by the ability of athymic mice to mount an effective immune response to trypanosome infection.

Using ^{75}Se-labelled trypanosomes to assess the immune response in CFLP and C57Bl mice to T. congolense infection, it was shown that CFLP mice were unable to remove a large percentage of the injected activity by hepatic uptake. These differences were confirmed by infectivity neutralisation and trypanolysis of radio-labelled 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. It has been shown that mouse complement is only poorly activated by IgM, while guinea pig serum is readily activated by IgM (Klaus, Pepys, Kitajima and Askonas, 1979). Thus the high trypanolytic activity of serum from infected C57Bl mice, in the presence of guinea pig serum 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.

Cyclophosphamide has been reported to enhance infections in mice (Luckins, 1969; Vickerman et al, 1977). It was found in this study that total body irradiation, splenectomy and pretreatment with cyclophosphamide all abolished the ability of C57Bl mice to control the peripheral parasitaemia and death occurred with a fulminating parasitaemia. The integrity of the host's immune response appeared, therefore, to be important in resistance to infection, and this was supported by infectivity neutralisation, trypanolysis and immune clearance assays.

In view of the evidence that antibody is essential in

controlling parasitaemia, 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 anti-trypanosome responses may be blocked (Oberbarnscheidt and Kolsch, 1978) or that levels of IgG, in the case of hyperimmune serum, inhibited the production of IgM and thus reduced the effectiveness of the immune response (Finkelstein and Uhr, 1964). Furthermore, *in vitro* assessment of protective ability does not take into account the possibility that antigenic variation may rapidly circumvent the protective ability of the serum given *in vivo* a few days after infection (Balber, Bangs, James and Proia, 1979). On the other hand, the experiments in Chapter 2 demonstrated that passive immunisation against the same parasite could be achieved with maternal antibody, which is of the same class (IgG) as that in hyperimmune serum. It may be therefore that the explanation for the failure to achieve passive immunisation with serum was that insufficient was used. Little information is available on this aspect, as most reports on passive immunisation have been concerned with *T. brucei rhodesiense* and *T. brucei gambiense* and the immune serum was always administered around the time of infection (Seed and Gam, 1966; Takayanagi, Kambara and Enriques, 1973a; Campbell and Phillips, 1976).

The susceptibility of CFLP mice could not be overcome by decreasing the number of infecting organisms, indicating that the

ability to mount an effective immune response is dependent upon an intrinsic mechanism, rather than the speed of the immune response. This was supported by the finding that a massive primary dose of non-replicative trypanosomes given simultaneously with a small viable infection was an insufficient stimulus to the immune system. It is possible, however, that a minor variant, not represented in the priming population, was responsible for the parasitaemia which eventually killed the host.

It has been proposed that non-specific stimulation of the MPS can alter the susceptibility of mice to T. congolense and T. brucei infections (Singer, Kimble and Ritts, 1964; Murray and Morrison, 1979). However, in the current study, only minimal benefit was conferred by E. coli LPS, in that 3 of 5 mice survived the initial peak of parasitaemia, only to succumb to a fulminating relapse parasitaemia. Thus, an increase of the activity of the MPS in the absence of effective levels of antibody did not reduce susceptibility to the particular parasite used in this study, although it is possible that with a trypanosome of less virulence the effect of non-specific stimulation may be more pronounced, which may account for the success of the previous study (Murray and Morrison, 1979).

The ability of immunostimulants to increase resistance to a number of other parasitic infections has now been extensively examined. In several instances they stimulated a dramatic increase in host resistance which in some cases was complete. In mice,

pre-inoculation with C. parvum has been shown to increase host resistance to Plasmodium spp. (Nussenzweig, 1967; Clark et al, 1977a; Cottrell, Playfair and de Sousa, 1977), Babesia spp. (Clark et al, 1977a) and Trypanosoma cruzi (Kierszenbaum, 1975; Brener and Cardoso, 1976). Similarly, BCG has also been used to stimulate increased resistance to Plasmodium spp. and Babesia spp. in mice (Clark, Allison and Cox, 1976; Clark, Wills, Richmond and Allison, 1977b), T. cruzi in mice (Ortiz-Ortiz, Gonzalez-Mendoza and Lamoyi, 1975), Leishmania donovani in mice (Smrkovski and Larson, 1977; Weintraub and Weinbaum, 1977), Schistosoma mansoni in hamsters and in mice (Capron and Lesoin, 1969; Fauve and Dodin, 1976; Bout et al, 1977; Civil, Warren and Mahmoud, 1978), Ecchinococcus multilocularis in cotton rats (Rau and Tanner, 1975), Ecchinococcus granulosus in the gerbil (Thompson, 1976), the systemic larval phase of Trichinella spiralis in mice (Grove and Civil, 1978) and Toxoplasma gondii in the rabbit (Tabbara, O'Connor and Nozik, 1975). On the other hand, Brocklesby and Purnell (1977) reported the failure of BCG to protect both splenectomized and intact calves against challenge with Babesia divergens.

Despite the failure to increase resistance in the current study with immunostimulants, this is an approach which deserves further investigation, particularly in the bovine, since if successful, the high productive qualities of certain susceptible breeds may be exploited if increased resistance can be stimulated.

In conclusion, the results in this chapter indicate that

the C57B1 mouse strain is inherently more resistant to T. congolense infection, through its ability to limit and reduce peripheral parasitaemia. Strains incapable of achieving this die from an uncontrolled fulminating parasitaemia. Several studies in laboratory animals have indicated that IgM is the important Ig class in controlling the parasitaemia and in protection (Campbell et al, 1978), there is evidence that this is the case in cattle (Luckins, 1976; Musoke, Nantulya, Barbet, Kironde and McGuire, in press), and the same is likely to be the explanation for the resistance of the C57B1 mouse.

CHAPTER IV

Radiolabelling of trypanosomes with [^{75}Se]-methionine and their use in studies on immunological clearance

INTRODUCTION

The immune response to pathogenic trypanosomes has been the subject of much investigation, but is still poorly understood. Considerable attention has been paid to the antibody response to infection and the apparent failure of this response to control infections because of the parasite's notable ability to undergo antigenic variation. There is evidence that macrophages and cell-mediated responses also play an active role in combating the infection (see review by Murray and Urquhart, 1977), thus quantitative evaluation of these responses *in vivo* would be aided by the availability of a suitable method for measuring the removal of circulating trypanosomes.

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 *Trypanosoma brucei*. Much work has been done in the use of radioactive isotopes for the labelling of cells for use in cell mediated immune reactions *in vitro*, and from this work have emerged the disadvantages of the various radiolabels in particular assay systems. Long term assays involving cytostatic and cytotoxic influences (Takasugi and Klein, 1970) or only cytotoxic effects (Bean, Pees, Rosen and Oettgen, 1973; Oldham and Herberman, 1973) are normally not suitable for short term reactions. Short term assays able to monitor close cell to cell contact (Henney, 1976) or immediate cytotoxic effects (Brunner, Maucl, Cerottini and Chapins, 1968) are usually not suitable for

long term assays, due to the high spontaneous release of radioactive material. The use of radioisotope-labelled target cells as an evaluation technique for various types of cell mediated immune reactions *in vitro* has become well established. Labelling with β-isotopes, such as ^{3}H, ^{14}C, or ^{45}Ca (reviewed by Rooijen, 1977) has two major disadvantages: β-particles are usually more efficient in cell-killing than γ-rays (Bedford, Mitchell, Griggs and Bender, 1975) and their preparation for counting is more laborious and expensive than that of γ-isotopes. Moreover, β-isotopes suffer from quench problems, particularly when dissolved in coloured media. More easily-detectable γ-isotopes can be incorporated into cells in different ways: $[^{125}I-]$ iododeoxyuridine ($^{125}IUdR$) is selectively taken up by DNA-synthesising cells (Nak and Till, 1963). Resting as well as proliferating cells can be labelled with various free γ-isotopes such as ^{51}Cr (Sanderson, 1964; Wigzell, 1965), ^{67}Ga (Burleson, Johnson, and Head, 1974), ^{86}Rb (Walker and Lucas, 1972), ^{99m}Tc (Gillespie, Barth and Gobuty, 1973) and ^{197}Hg (Saha, Schell and Farrer, 1977).

Several attempts to label trypanosomes with radioisotopes 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; Viens and Targett, 1972; Sanderson, Bunn and

Lopez, 1978). [^{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 apparently not incorporated into T. vivax (Isoun and Isoun, 1974) presumably due to the parasite's ability to synthesise thymidine de novo.

The gamma emitting isotope [^{51}Cr]-chromium has been used successfully in cytotoxicity tests (Batchelor, 1973), and not surprisingly attempts have been made to label parasitic organisms with this radioisotope. Schistosoma mansoni schistosomula have been successfully labelled (Butterworth, Sturrock, Houba and Rees, 1974) and ^{51}Cr -labelled Litomosoides carinii microfilaria have found successful use in a cytotoxicity assay (Subrahmayan, Rao, Mehta and Nelson, 1976). In contrast, attempts to label trypanosomes with [^{51}Cr]-chromium have been less successful. The labelling efficiency of T. cruzi was low even when beta particle emissions were measured by liquid scintillation in preference to gamma emissions (Kuhn, Vaughn and Ianuzzi, 1974).

The use of [^{99m}Tc]-technetium to label T. dionisii (Mkwanazi, Franks and Baker, 1976) was limited by the isotope's short half-life of 6 hours and difficulties in its manufacture from [^{99}Mo]-molybdenum. Two major criticisms arise when using in vitro cultivation to label trypanosomes. First, the incubation may result in a possible alteration to the parasite's surface antigen profile and secondly the incubation may adversely

affect the parasite's subsequent infectivity and replication in the host (Dahlin, Hungerer and Zwischer, 1976). A further limitation is on the use to which labelled parasites may be subsequently put, since a reagent synthesised from [^{35}S]-methionine (Cross, 1975), and the procedure using ^{125}I or ^{3}H described by Rovis, Barbet and Williams (1978) label only the surface coats of T. brucei and T. congolense respectively, the reagents being unable to penetrate the cell membrane.

The problems associated with in vitro incubation may be overcome if the labelling is carried out in vivo, particularly if the isotope is metabolically incorporated into the trypanosome. Thus, while ^{3}H pyrimidines have been used in the past, a radiolabelled gamma-emitting amino acid analogue was used, to overcome the problem of sample preparation for liquid scintillation yet benefit from the isotope's metabolic incorporation.

This chapter describes a series of experiments designed to investigate the possibility of labelling trypanosomes with [^{75}Se]-methionine and 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 (Appendix I).

Experimental Animals. These were female Hooded Lister rats (Olac 1976 Ltd., Bicester), female CFLP mice (Anglia Laboratory Animals, Huntingdon), and AKR strain mice genetically deficient in the 5th component of complement (C5) (kindly donated by Dr. I. McConnell, MRC, Cambridge. Prior to their use the absence of C5 in AKR mice was confirmed by haemolytic radial diffusion (Lachman and Hobart, 1978). Animals which were 8-10 weeks of age at the beginning of each experiment were used throughout.

Radiolabelling technique. Radiolabelled trypanosomes were prepared in rats with fulminating parasitaemias, normally 4 or 5 days after infection. The rats were sublethally irradiated 1 day prior to infection with 650 rad in a ^{60}Co source (Nuclear Engineering Ltd., Berkshire). 50 µCi $\left[^{75}Se\right]$-methionine (Radiochemical Centre, Amersham : 100 µCi/ml containing 10-50 µg methionine/ml) was injected i.v, the infected blood collected 20 hours later, and the trypanosomes separated from blood cells by DEAE cellulose chromatography (Lanham and Godfrey, 1970).

Injection of radiolabelled trypanosomes and sampling. Washed radiolabelled trypanosomes were diluted in PSG to give a final concentration of 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 Fentanyl base (0.2 mg/ml) and Fluarisone (10.0 mg/ml) (Hypnorm, Janssen Pharmaceuticals, Belgium). After 1 hour, unless otherwise stated, the animals were given an over-dose of trichloroethylene and a blood sample taken by cardiac puncture. The spleen and liver were carefully excised, washed in PSG, blotted dry and weighed. Spleen, liver and an aliquot of blood (0.5 ml) were placed in individual counting vials, the remainder of the carcase was divided into a further 3 counting vials, and the radioactivity determined using an automatic gamma scintillation counter (Packard tri-carb 3330, Illinois, USA).

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.

Immunisation procedure. Mice were immunised by infection and trypanocidal therapy using diminazene aceturate (Berenil, Hoechst, Germany; 40 mg Active Principle/kg body weight). The drug was

administered i.p. in distilled water on the fourth day of infection, and 4 weeks later the mice were challenged with the homologous strain of parasite. Failure to detect parasites over the ensuing 30 days was taken as evidence of immunity. Passive immunisation was done by i.v. injection of 0.2 ml undiluted hyperimmune serum 15 minutes after the injection of ^{75}Se -labelled trypanosomes.

Preparation of Hyperimmune serum. Hyperimmune serum (HIS) was obtained from rats infected with T. brucei 226 and subsequent drug cure with Berenil (40 mg/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.

Homogenization and Protein Separation of ^{75}Se -labelled Trypanosomes. A 5 ml sample of ^{75}Se -labelled trypanosomes containing approximately 5×10^8 trypanosomes/ml PSG was disintegrated by 5 cycles of freezing in liquid nitrogen and thawing to 37°C in a temperature-controlled water bath. 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 eluant 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 Whatman's 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 on either side. The sample was then run for 16 hours at 100 volts. The strip was removed, dried and stained in 0.1% bromophenol blue (George T. Gurr, London), dissolved in methanol for 20 min. It was then washed with dilute acetic acid to remove superficial stain, and 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) 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% Ponceua 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 Folin phenol method of Lowry, Rosebrough, Farr and Randall (1951) or by measuring the solution at 260 nm and 280 nm 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 a homogenised sample. The resultant precipitate was sedimented in a temperature controlled centrifuge

at 1500 g for 20 min at 4°C. The percentage protein-bound radioactivity was calculated as follows:-

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

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 1×10^8 trypanosomes. They were then maintained at 37°C.

Decomplementation of mice. Mice were decomplemented by 3 i.v. injections of purified cobra venom factor (CVF) over a period of 24 hours. Each mouse received a total of 25 µl CVF. The ability of these mice to remove radiolabelled trypanosomes was determined 24 hours 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., Berkshire).

Stimulation of the mononuclear phagocytic system (MPS). Non-specific stimulation of the 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.

In vitro treatment of trypanosomes with serum. (a) Neutralising antibody infectivity tests were performed on sera as described by Lumsden et al (1973) with the following minor alterations. The test was performed in 1 ml plastic tubes. Each tube contained 200 µl test serum or PBS, 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 $4^{\circ}C$ for 30 min 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) Agglutination titres of fresh HIS were determined in microtitre plates (Cooke Laboratory Products, Virginia, USA). 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 $37^{\circ}C$ for 30 min and then read on an inverted microscope (Leitz, Germany) at X 400 magnification.

(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 $4^{\circ}C$ for 30 min in HIS or normal rat serum (NRS) without guinea pig complement.

Some of the trypanosomes were then washed 3 times with PSG.

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

Statistics. Variation around the mean is expressed as the standard error. Unless otherwise stated, groups of mice comprised 5 to 8 individuals.

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 hr later, respectively.

The results presented in Table 4.1 show that the shorter the exposure of the parasite to the isotope the greater the labelling efficiency, i.e. 0.04% after 20 hr 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 done:

(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.

Table 4.1 The relationship between specific activity and labelling time

Period of incubation	c.p.m. per 10^8 trypanosomes	% labelling efficiency*
20 hr	4053 $\pm$ 259	0.04
5 days	450 $\pm$ 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.

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 hr later. The specific activity of these parasites is presented in Table 4.2.

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, Adelstein 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) 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

Table 4.2 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

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 4.1 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 a 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 4.3 show that the majority of the ^{75}Se-activity in the serum from normal rats given ^{75}Se -

Fig 4.1

Chromatographic separation on DEAE-cellulose of a homogenate of ^{75}Se-methionine-labelled T. brucei.

(– – – – –) ^{75}Se radioactivity

(· · · · · · · · · · · ·) absorbance at 260nm

(————————) absorbance at 280nm

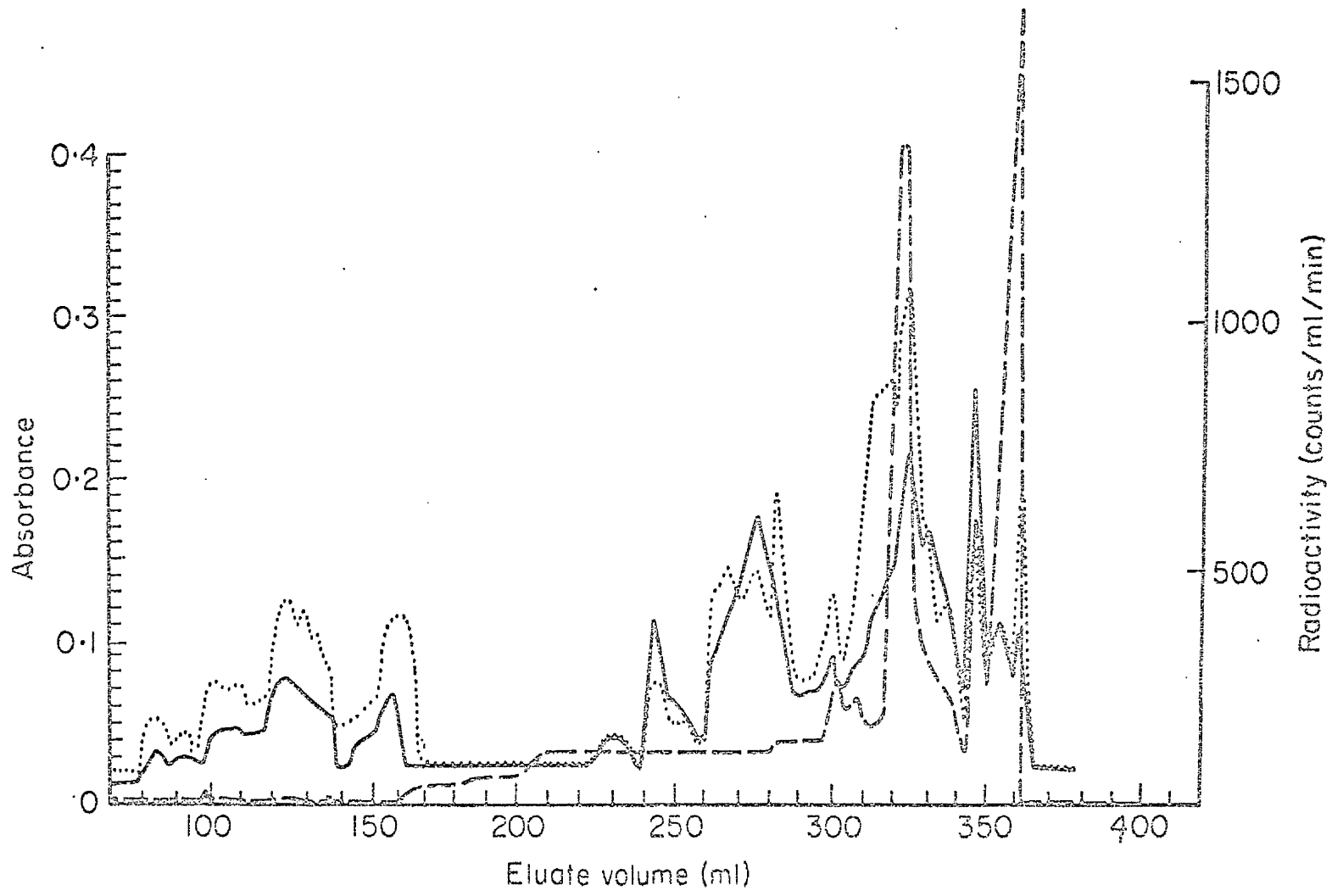

0·4
0·3
0·2
0·1
0
Absorbance
1500
1000
500
Radioactivity (counts/ml/min)
100
150
200
250
300
350
400
Eluate volume (ml)

Table 4.3 Paper electrophoresis of serum proteins from a normal rat given 30 μCi of [^{75}Se]-methionine 20 hr previously

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

Table 4.4 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

methionine 20 hr previously can be located in the globulin component. In addition, when ^{75}Se-labelled proteins from a homogenate of washed trypanosomes and host plasma proteins were run in parallel on cellulose acetate, the results showed (Table 4.4) that the majority of trypanosome ^{75}Se-activity was associated with a band corresponding to the serum albumin of host plasma proteins. In contrast, the majority of plasma protein ^{75}Se-activity is against 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 1×10^8 organisms. These bottles were then incubated at 37°C for between 1 hr and 4 hr. 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 4.5 show that *in vitro* leakage of ^{75}Se-activity is approximately 10% after 4 hr.

(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 1×10^8 radiolabelled trypanosomes. These animals were then killed 1 hr or 4 hr 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 results presented in Table 4.6 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 4.7 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 hr.

Table 4.5 Elution of radioactivity during *in vitro* incubation of ^{75}Se -labelled *T. brucei*

Time (hr)	% 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 4.6 The estimated total blood radioactivity 1 hr and 4 hr after the infection of ^{75}Se -labelled T. brucei

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

Table 4.7 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 hr	2600 ± 193	67.4

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:-

A homogenate prepared from ^{75}Se-labelled trypanosomes (4328 $\pm$ 67 cpm) 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 $\pm$ 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 hr later by DEAE cellulose chromatography of the infected blood.

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 hr 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 min. 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 4.2. 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 min 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 min 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 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.

Fig 4.2

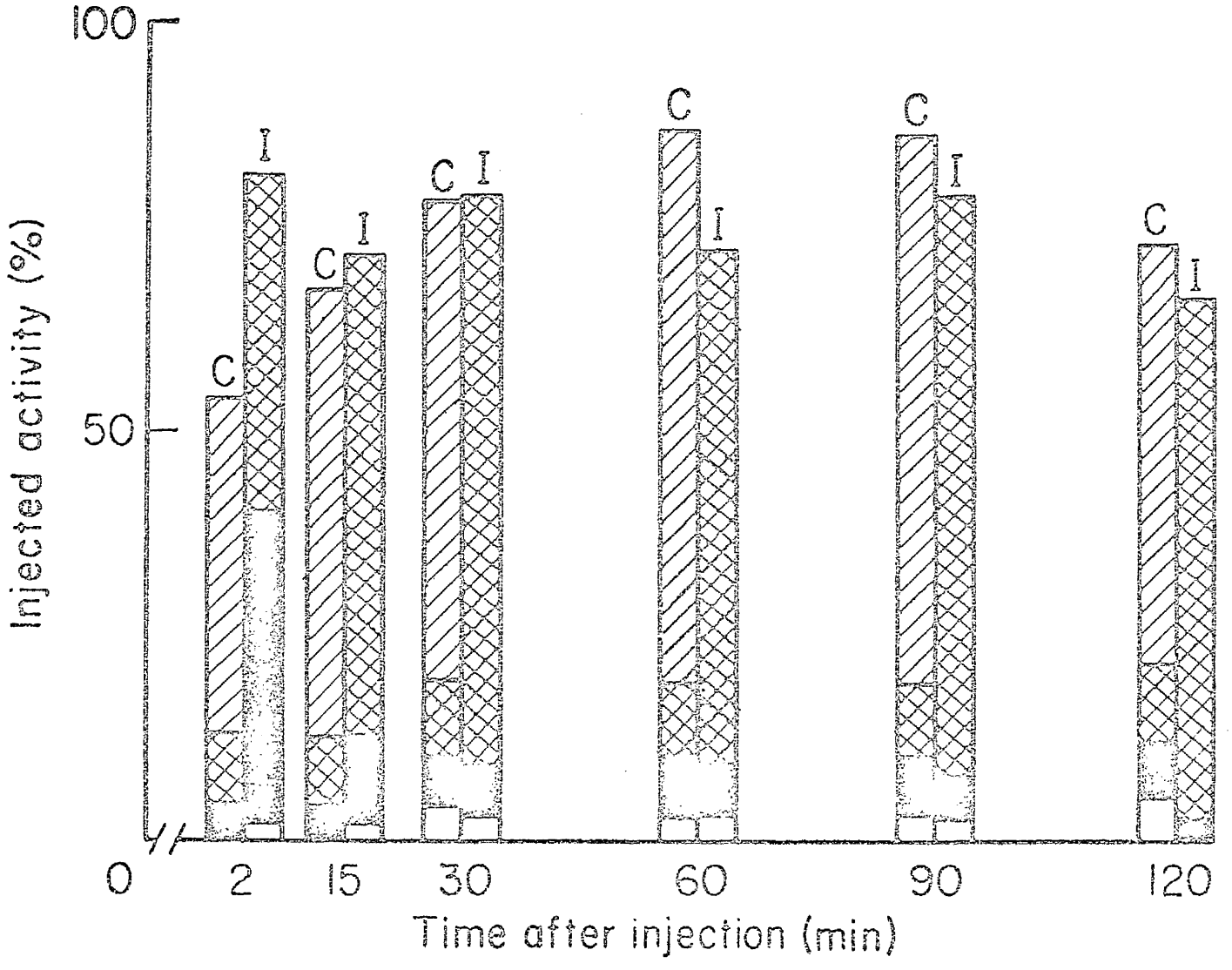
The tissue distribution of ^{75}Se-labelled trypanosomes at intervals after injection into immunised (I) and control (C) mice.

 : lungs

 : spleen

 : blood

 : liver


100
50
0
Injected activity (%)
C
I
2
15
30
60
90
120
Time after injection (min)

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 $\left[^{75}\text{Se}\right]$-methionine by i.v. injection and 1 hour later the tissue distribution of ^{75}Se-activity recorded. Table 4.8 shows that the tissue distribution of $\left[^{75}\text{Se}\right]$-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.

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 above, was principally

Table 4.8 Tissue distribution of $[^{75}Se]$-methionine in normal and immune mice

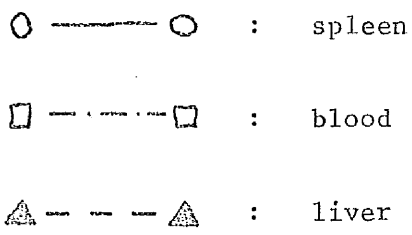
	tissue distribution (% injected activity)	
	spleen	liver
Control	< 3.0	16.5 ± 1.6
Immune	< 3.0	15.7 ± 1.1

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 min later by various i.v. doses of HIS. The results presented in Fig 4.3 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 4.9 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.

Fig 4.3

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



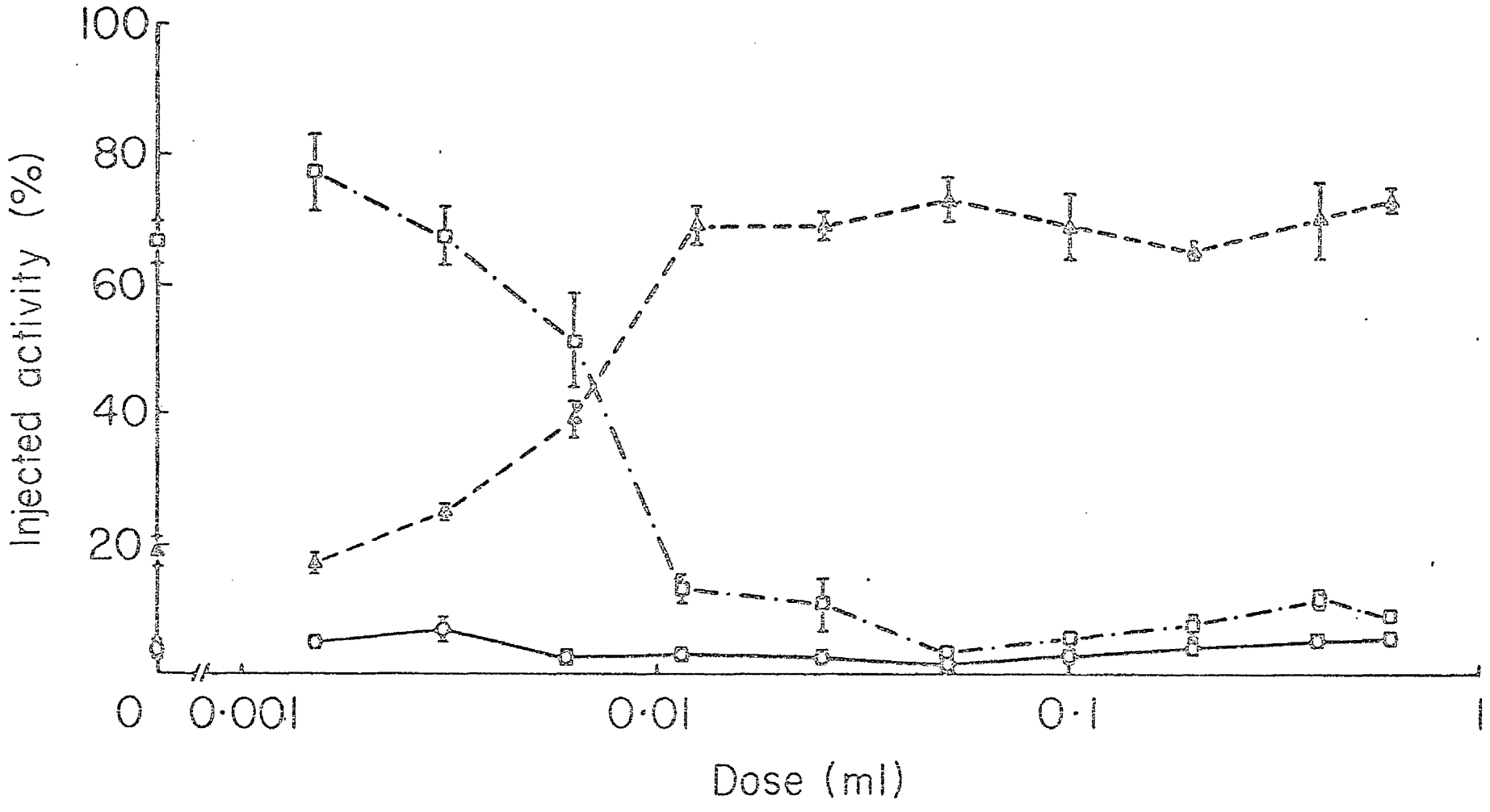

100
80
60
40
20
0
Injected activity (%)
0·001
0·01
0·1
1
Dose (ml)

Table 4.9 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

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 immuno-suppressed 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 4.10 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 min later with

Table 4.10 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

Table 4.11 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

0.2 ml of a 1:8 dilution of HIS. The results presented in Table 4.11 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 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, Murray, Jennings, Fisher and Urquhart, 1974; 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.

To test this, 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 bacterial cells (Stiffel, Mouton and Biozzi, 1970).

The results presented in Table 4.12 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 Table 4.9).

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, the following experiments were done.

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 min 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 4.13 show that C5 deficient mice had hepatic uptakes only slightly

Table 4.12 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

Table 4.13 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 4.14 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

lower than those of passively immunised 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 4.14 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 a control value of approximately 9%.

DISCUSSION

L-selenomethionine is a rare natural analogue of the more common sulphur-containing methionine, and since there is only little difference in the uptake of both amino acids *in vivo* and in tissue culture (Pan, Natori and Tarver, 1964), [^{75}Se]-selenomethionine (Blau and Bender, 1962) has been routinely employed for more than 15 years in clinical diagnosis in animals and in humans (Spencer, Montana, Scanlon and Evans, 1967; Herrera, Gonzalez, Schwartz, Diggs and Belsky, 1965) and 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 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 trypano-

somes, 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 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; 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 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 remains 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 hr.

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 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, Jennings, Murray and Urquhart, 1974), 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 trypanolysis, 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.

Thus, 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.

Thus by 60 minutes 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 4.3, 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 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 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 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.

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.

Appendix I

The trypanosomes used in the experiments described in this thesis are described below.

A. 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 infected cow in June 1961 at the East African Trypanosome Research Organisation, Tororo 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, 3 days
TREU 87
↓ 3 days
TREU 164
↓ 3 days
TREU 226

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

TREU 667 was originally isolated during the Edinburgh Veterinary Expedition of 1966 in Busoga district, Uganda. The primary isolate was designated Busoga/66/EVE/10, and was from a naturally infected cow.

TREU 62 ≡ Busoga/66/EVE/10
↓ 22 d (rats)
TREU 667

This parasite produces an infection in mice of 6-12 weeks and in rats 5-8 weeks.

The third Trypanosoma brucei brucei stabilate was originally provided by Professor W.H.R. Lumsden, London School of Tropical Medicine, and was numbered LUMP 1001 (London University Medical Parasitology). The primary isolate S10 was from a naturally infected wildebeest in the Serengeti in 1966.

S10
↓ 6, 12 days rat
stabilate
↓ 3 days mouse
LUMP 22
↓ 3 days mouse
LUMP 43
↓ 42 days mouse
LUMP 68
↓ 10 days mouse
LUMP 1001

This parasite produces in CFLP mice an infection of 4-6 weeks' duration.

B. Nannomonas. This parasite was kindly provided by Professor K. Vickerman as Glasgow University Protozoology (G.U.P.) 93. The origin of this stabilate is unknown, and was described as T. congolense on the basis of its morphology. T. congolense GVR 1 (Glasgow Veterinary Research) was a working stabilate derived from this after 4 days in irradiated mice (650 rad). T. congolense GVR 1 produces 2 distinct patterns of parasitaemia (Chapter 3) depending on the strain of its host. In CFLP mice the infection is acute and fulminating and causes death in 8-12 days, whereas in C57Bl mice a chronic infection associated with periods of remission and relapsing parasitaemia results.

From all of the above stabilates, which themselves were kept in liquid nitrogen as reserve stabilates, working material was grown up in irradiated mice and cryopreserved in liquid nitrogen in silicon tubing (Portex Ltd., Kent) using glycerol as the preservative at a final concentration of 10% (v/v) (Taylor, 1972). This material was used in all experiments to initiate infections.

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