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Immunological clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice

III. STUDIES IN ANIMALS WITH ACUTE INFECTIONS

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Summary. Using trypanosomes labelled with [^{75}Se]-methionine a series of experiments was conducted to investigate antibody production in mice with acute fulminating *T. brucei* infections. As measured by the hepatic uptake of radiolabelled parasites, we were unable to demonstrate any evidence of antibody-mediated uptake by the liver in such mice.

It was concluded that this was not due to impaired macrophage function but was caused by the inability of antibody production to cope with the massive parasitaemias produced by rapidly-replicating infections so that effective opsonization of the parasites did not occur. In contrast, a strain of trypanosome which causes a more chronic infection, although initially having a similar replication rate, subsequently switched to a slower one and thereby allowed antibody to reach levels which permitted effective opsonization.

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

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INTRODUCTION

Although African trypanosome infections of man and domestic animals are characteristically sub-acute or chronic, acute fulminating infections are not uncommonly observed (Apted, 1970; Fiennes, 1970; Chizyuka, 1980). The pathogenesis of such infections has not been fully evaluated but may depend on a number of factors such as the strain of parasite, the size of inoculum (Murray & Morrison, 1979), the rate of replication (Soltys & Woo, 1969), the degree of biochemical derangement (Moon, Williams & Witherspoon, 1968) and the release of haemolytic toxins by the parasite (Chi, Webb, Lambert & Miescher, 1977). However, a principal factor in determining the course of trypanosome infections is probably the effectiveness of the host's immune response. This is thought to depend largely on antibody although there is also evidence that macrophages and complement may play important contributory roles (see MacAskill, Holmes, Whitelaw, McConnell, Jennings & Urquhart, 1980).

However, it has been recently suggested from studies in mice that the generalized immunosuppressive effects associated with trypanosome infections may impair the full expresssion of the host's antibody response to the parasite (Hudson & Terry, 1979). Indeed the degree to which strains of trypanosomes possess this immunosuppressive effect has been suggested as the primary basis for parasite virulence (Sacks, Selkirk, Ogilvie & Askonas, 1980).

The development of a method for labelling trypanosomes with [^{75}Se]-methionine (MacAskill, Holmes, Whitelaw, Jennings & Urquhart, 1978; Holmes, MacAskill, Whitelaw, Jennings & Urquhart, 1979) has facilitated evaluation of the immune response *in vivo*. From such studies it has been shown that mice either actively or passively immunized against a stabilate of *Trypanosoma brucei* rapidly clear homologous radiolabelled parasites from their circulation essentially as a result of antibody-mediated hepatic uptake (Holmes *et al.*, 1979; MacAskill *et al.*, 1980).

The present studies were designed to evaluate the effectiveness of the immune response *in vivo* of mice with acute fulminating infections of *T. brucei* using ^{75}Se-labelled parasites.

MATERIALS AND METHODS

Parasites

A single stabilate of *T. brucei* derived from a stock of TREU 226 was used throughout the study except in two experiments where a stabilate of *T. brucei* derived from a stock of TREU 667 was also used. *T. brucei* 226 causes a fulminating parasitaemia which is fatal in 5 to 7 days following infection in mice and rats, while *T. brucei* 667 causes a moderate parasitaemia extending over several weeks. Mice and rats were infected with 1×10^4 and 1×10^5 organisms, respectively, by intraperitoneal (i.p.) injection. Parasitaemia was estimated using the technique described by Herbert & Lumsden (1976)

Laboratory animals

These were CFLP mice weighing 20 to 30 g and female Hooded Lister rats weighing between 130 and 180 g. A minimum of four animals per group were used in each experiment.

Irradiation of laboratory animals

When necessary mice and rats were sub-lethally irradiated 1 day before infection with 650 rad in a ^{60}Co source.

Radiolabelling techniques

Radiolabelled trypanosomes were prepared using [^{75}Se]-methionine by an *in vivo* labelling method (Holmes *et al.*, 1979). The trypanosomes were separated from infected blood using a DEAE cellulose (Whatman Chromedia DE52) column by the method described by Lanham & Godfrey (1970). The suspension of washed ^{75}Se-labelled trypanosomes was diluted with phosphate glucose buffered saline (PGBS), pH 8·0 to give an injection dose of 1×10^8 organisms (approximately 4000 c.p.m.) per mouse.

One hour after the intravenous (i.v.) injection of labelled parasites the mice were killed and the distribution of radiolabelled parasites in various organs determined as previously described (Holmes *et al.*, 1979).

Measurement of replication times of two stabilates of T. brucei

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

Preparation of hyperimmune serum

Hyperimmune serum (HIS) was obtained from rats infected with *T. brucei* 226 and subsequent drug cure with diminazine aceturate (40 mg/kg Berenil, Farbwerke, Hoechst) 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.

Vaccination of mice

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

Groups of mice were then vaccinated with either *T. brucei* 226 or *T. brucei* 667 by i.v. injection of 2×10^8 irradiated trypanosomes.

IgM plaque-forming cell assay (PFC)

Mice were primed with 1×10^8 sheep red blood cells (SRBC) and the direct (IgM) anti-SRBC response assayed 5 days later by a modification of the Jerne technique (Cunningham & Szenberg, 1968). The assay was performed on pooled spleen cells obtained from groups of four mice. Each test well contained 150 μl of a spleen cell suspension, 10 μl guinea-pig serum and 20 μl of a 15% suspension of SRBC.

In vitro *treatment of trypanosomes with serum*

(a) Neutralizing antibody infectivity tests were performed on sera (Lumsden, Herbert, & McNeillage, 1973). The test well contained 200 μl serum, 50 μl guinea-pig serum and 5×10^4 trypanosomes/250 μl PGBS. After 30 min at 4°C the contents of the test well were taken up in a syringe and five normal mice each inoculated i.p. with an equal aliquot of the test well. The mice were then monitored daily for the appearance of parasites by wet blood film examination.

(b) Clearance studies of labelled trypanosomes were conducted following their *in vitro* treatment with serum. This consisted of a modified infectivity neutralization test in which ^{75}Se-labelled trypanosomes were incubated at 4° for 30 min in HIS or in normal serum. The trypanosomes were then washed three times with PGBS. 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.

RESULTS

Clearance of ^{75}Se-labelled *T. brucei* 226 by acutely infected mice

In this experiment the ability of highly parasitaemic mice, first infected 4 days previously, to remove labelled homologous trypanosomes was investigated. The results of this experiment present in Table 1 showed that such mice did not differ appreciably from uninfected control mice in that they were unable to remove radiolabelled trypanosomes and most of the injected activity remained in the blood. These results suggest that acutely-infected mice had inadequate levels of antibody or impaired phagocytic function.

Table 1. The tissue distribution of radiolabelled homologous trypanosomes injected 1 hr previously into mice with a fulminating parasitaemia of *T. brucei* 226

	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
Infected mice	5·5±0·5	14·1±0·6	69·8±3·7
Uninfected mice	3·0±0·4	12·4±0·6	83·8±4·8

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

The effect of passive immunization on the clearance of labelled trypanosomes by infected mice.

In order to investigate if the failure of mice infected with *T. brucei* 226 to remove radiolabelled trypanosomes from their circulation was due primarily to an absence of antibody, a group of trypanosome-infected mice was given HIS i.v. Since previous studies have demonstrated the efficacy of passive immunization with relatively small amounts of HIS (0·01 ml) in promoting high hepatic uptake in normal mice (MacAskill *et al.*, 1980), a single intravenous injection of HIS (0·2 ml) was administered to mice infected 4 days previously, 15 min after the injection of radiolabelled trypanosomes. The results in Table 2 show that this amount of HIS failed to increase hepatic uptake in such animals, yet it was highly effective in inducing blood clearance of radio-labelled parasites in normal mice.

Two possible explanations for this result are first, that the quantity of HIS was insufficient to effectively opsonize the large numbers of circulating parasites or, secondly, it may be that the numbers of available phagocytes were reduced as a result of their saturation by the heavy parasitaemia.

The functional integrity of the MPS in highly parasitaemic mice

In an attempt to assess the availability of hepatic macrophages for trypanosome removal in highly parasitaemic mice infected 4 days previously, groups of mice received labelled *T. brucei* 226 which had been incubated in HIS before their injection. The results in Table 3 showed that pretreatment of the labelled trypanosomes enabled the parasitaemic mice to remove a large proportion of the labelled parasites from their circulation by hepatic uptake, i.e. approximately 62% as compared with 15% in control mice. Thus a high parasitaemia *per se* does not impair the ability of macrophages to remove trypanosomes provided they are suitably opsonized.

The results of these three experiments indicate that the inability of acutely infected animals to remove trypanosomes from their circulation is due to a failure

Table 2. The effect of passive immunization on the tissue distribution of ^{75}Se-labelled *T. brucei* in mice with a fulminating parasitaemia

Group	Hyperimmune serum (ml)	Tissue distribution (% injected activity) Spleen	Liver	Blood
Infected	0	4·4±0·6	12·4±0·7	81·4±4·4
Infected	0·2	6·1±0·9	15·1±1·6	73·6±3·3
Uninfected	0	<3·0	10·2±0·8	83·9±5·3
Uninfected	0·2	<3·0	62·1±3·5	12·4±2·1

to achieve adequate levels of circulating antibody. This may be either a result of impaired antibody production associated with trypanosome-induced immunosuppression or parasite replication 'outpacing' antibody synthesis and thereby leading to a relative deficiency of antibody despite increased production.

The relationship between parasitaemia and circulating antibody

To find if the presence of large numbers of replicating trypanosomes prevents mice achieving effective levels of circulating antibody, Berenil, a drug which inhibits trypanosome replication, was administered to groups of infected mice 5 hr and 24 hr before the injection of labelled parasites. The results are presented in Table 4.

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

The examination of wet blood smears of infected blood 5 hr post therapy showed the presence of large numbers of parasites, however, by 24 hr there was a dramatic reduction: the parasitaemia falling from 10^9 trypanosomes/ml blood to less than 10^6 trypanosomes/ml blood.

The results might suggest that in rapidly-replacing infections of trypanosomes, antibody is being produced but not in sufficient quantities to promote obvious opsonization and hepatic uptake. However, if replication is stopped effective antibody levels are achieved within 24 hr.

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

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

Table 3. The tissue distribution of ^{75}Se-labelled homologous *T. brucei* previously incubated in hyperimmune serum before injection into highly parasitaemic mice

Group	Opsonized trypanosomes	Tissue distribution (% injected activity) Spleen	Liver	Blood
Infected	−	5·8±0·8	14·7±0·6	61·1±14·2
Infected	+	<3·0	61·9±2·7	8·7±2·1
Uninfected	+	<3·0	70·9±1·6	<3·0

Table 4. The tissue distribution of ^{75}Se-labelled homologous trypanosomes injected into groups of mice which had been infected with *T. brucei* 226 5 days previously and treated with Berenil before injection of parasites

Group	Interval (hr) between Berenil treatment and injection of radiolabelled trypanosomes	Tissue distribution (% injected activity)		
		Spleen	Liver	Blood
Infected	5	<3·0	10·8±0·5	74·4±0·2
	24	<3·0	56·5±1·6	6·0±0·5
Uninfected	24	<3·0	10·7±0·5	80·4±3·5

The results based on four groups of six mice and presented in Fig. 1 show that, whilst *T. brucei* 226 infection has a sustained single phase rate of growth, *T. brucei* 667 was characterized by a biphasic growth rate. During the initial period of growth, both strains had similar replication rates of approximately 5·5 hr. However, during the critical period between 5 and 7 days post-infection, the rates of replication were markedly different. In the acute infection (*T. brucei* 226) the replication rate remained constant and the mice died by day 5. In contrast, in the chronic infection (*T. brucei* 667) the rate of replication between day 5 and 7 slowed down markedly in both irradiated and non-irradiated mice to 37 and 18 hr, respectively.

Antibody was apparently essential for the control of the *T. brucei* 667 infection as irradiated animals died between days 7 and 8. It is also noteworthy that the parasitaemias were consistently higher in irradiated mice than in non-irradiated animals infected with *T. brucei* 667.

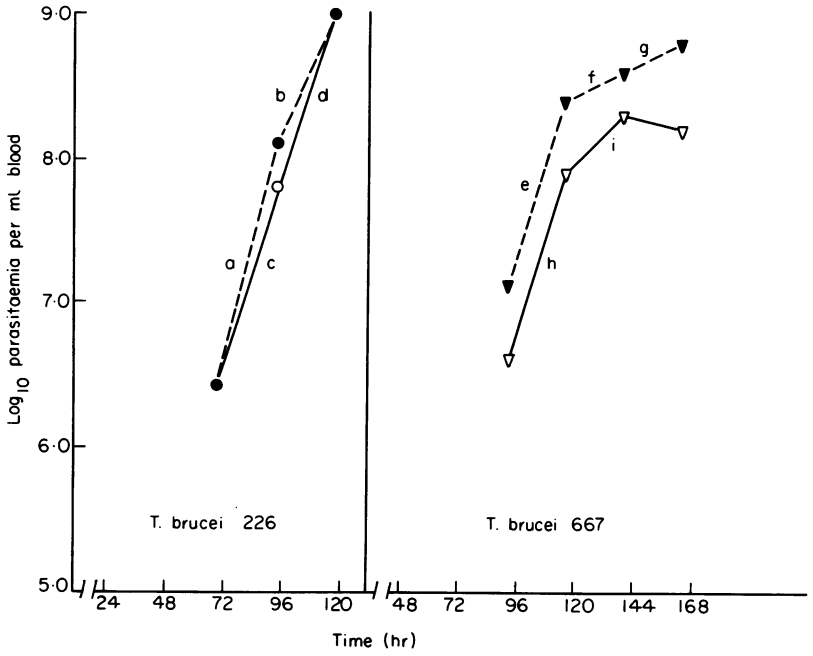

Figure 1. Parasitaemic profiles of *T. brucei* 226 and 667 in normal (——) and irradiated (- - - - -) mice. Replication rates obtained by regression analysis are (a) 4 hr; (b) 7·9 hr; (c) 5·2 hr; (d) 5·9 hr; (e) 5·9 hr; (f) 37·2 hr; (g) 36·3 hr; (h) 5·4 hr; (i) 18·4 hr.

Clearance studies (unpublished data) using ^{75}Se-labelled parasites showed that during the rapid growth phase (day 5) mice infected with either stock of *T. brucei* were incapable of removing significant numbers of labelled parasites from the circulation. However, mice infected with *T. brucei* 667 for 9 days could remove large numbers of parasites by hepatic uptake (42.7±3·8%) compared with control animals (11·5±0·4%).

The IgM PFC response to SRBC in parasitaemic mice primed on the day of infection

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

The results presented in Table 5 show that IgM antibody production to the SRBC at 5 days post-infection was as high as those of uninfected controls. There was, therefore, no evidence to suggest that IgM antibody synthesis was impaired in acutely-infected mice to an antigen presented contemporaneously with the trypanosome infection. *A priori*, it therefore seems probable that antibody production against the first parasitaemic peak would be similarly unimpaired.

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

In order to directly evaluate anti-trypanosome antibody production in infected mice, groups of animals were vaccinated with irradiated trypanosomes of one stock of *T. brucei* on the same day as infection with non-irradiated organisms of another *T. brucei* stock. The effectiveness of vaccination was then assessed 5 days later by the measurement of immune clearance of radiolabelled parasites of the same stabilate as that used for vaccination. The experimental design and results are presented in Table 6.

The results clearly show that neither infection with *T. brucei* 226 nor *T. brucei* 667 prevented successful vaccination against the other stabilate as judged by high hepatic clearance rates. These results therefore confirm that anti-trypanosome antibody production is not significantly impaired in acutely-infected mice.

Table 5. The PFC response of acutely-infected mice immunized with 1×10^8 SRBC on the day of infection and measured 5 days later

Group	SRBC given day of infection	Anti-SRBC PFC per spleen*
Infected	+	77,388±968
	−	<500
Uninfected	+	71,398±4609
	−	<500

* Average of six chambers.

Table 6. The tissue distribution of ^{75}Se-labelled trypanosomes in mice vaccinated with irradiated trypanosomes of one stock and simultaneously infected with another stock

Day 0		Day 5 tissue distribution (% injected activity)		
Vaccination	Infection	Spleen	Liver	Blood
		^{75}Se-*T. brucei* 226		
226	667	3·7±0·1	60·2±5·7	8·7±2·8
226	—	3·4±0·4	60·6±6·1	6·4±2·4
—	667	3·0±0·1	10·6±0·8	74·2±3·2
—	—	<3·0	12·4±0·6	74·5±1·9
		^{75}Se-*T. brucei* 667		
667	226	3·6±0·6	64·8±2·3	7·7±1·6
667	—	<3·0	61·4±1·2	4·8±1·0
—	226	8·2±1·3	29·5±2·9	65·3±3·8
—	—	5·0±1·3	15·2±2·9	64·4±2·1

DISCUSSION

Trypanosomiasis of man and animals is typically characterized by relapsing parasitaemias with antibody directed against each successive antigenic variant. However, infections where death is associated

with a massive parasitaemia some days after the first appearance of parasites in the blood are not uncommon. This paper is concerned with an investigation of the latter type of infection in an experimental model and, in particular, with the reasons underlying the apparent failure of the immune response. The parasite selected for these studies was *T. brucei* TREU 226 which, after inoculation into mice with 1×10^4 organisms, can first be detected in the blood after 2–3 days; thereafter their numbers rapidly increase so that at the point of death 2 days later 10^9 trypanosomes/ml are present.

Using the hepatic uptake of ^{75}Se-labelled trypanosomes as an index of the immune response (Holmes *et al.*, 1979; MacAskill *et al.*, 1980), we were unable to demonstrate the presence of any antibody-mediated uptake by the liver in mice 4 days after infection. This was not due to any failure of the MPS in such mice since labelled trypanosomes incubated *in vitro* with HIS and injected into similarly infected mice were rapidly cleared from the circulation. However, passive immunization of acutely-infected mice with HIS immediately following the injection of radiolabelled trypanosomes failed to induce to any significant extent their hepatic uptake. These results suggested that the very large numbers of parasites in the circulation 4 days after infection were such that the available antibody was insufficient to achieve effective opsonization and, hence, hepatic uptake. This possibility was supported by the results of an experiment in which acutely parasitaemic mice were treated with a trypanocidal drug 24 hr before the injection of radiolabelled trypanosomes. During this period the parasitaemia had fallen from 10^9 to less than 10^6 trypanosomes/ml and after the injection of labelled parasites their hepatic uptake was similar to that obtained in immune mice (MacAskill *et al.*, 1980).

A comparison of the replication rate of *T. brucei* 226 with that of *T. brucei* 667 which causes a chronic infection, showed that the virulent 226 had a constant replication rate of 5·5 hr between the time of first detection in the blood on day 3 and death on day 5. In the relapsing strain 667, the replication rate between day 4, when parasites were first detected in the blood, and day 5 was 5·4 hr but thereafter decreased to 18·4 hr, so that the peak parasitaemia of 667 occurred 1 day later and did not reach the level of 226.

Two aspects of the parasitaemic profile of 667 are of interest. First, the change in replication rate which occurred 120 hr after infection was also observed in sub-lethally irradiated mice indicating that it was not antibody-mediated but was an inherent characteristic of the strain and possibly associated with the development of intermediate and stumpy forms which occur towards the peak of parasitaemia in relapsing strains (Hoare, 1970). The second aspect is the actual decrease in circulating parasites which occurred after day 6. This could be reasonably attributed to the appearance of adequate amounts of antibody since it did not happen in irradiated mice, whose parasitaemia continued to increase until death 1 or 2 days later.

Recently Sacks *et al* (1980) have proposed that the virulence of different trypanosome strains, as measured by survival times, is directly correlated with their ability to induce immunosuppression. The latter was determined on the basis of altered PFC responses to SRBC in mice inoculated with membrane fractions of different strains of trypanosomes. While we accept that immunosuppression may be an important factor in the survival of animals with sub-acute or chronic infections, we were unable to show that it occurred in the acute infections studied by us. Thus firstly, mice inoculated with SRBC at the time of infection with 226 produced PFC responses comparable with those of uninfected mice. Secondly, mice simultaneously vaccinated with irradiated trypanosomes of one stock of *T. brucei* and infected with another stock at the same time developed, by day 5, levels of opsonic antibody comparable with those of vaccinated controls. Neither of these results would be expected in immunosuppressed mice.

We conclude that the acute fatal infections of trypanosomiasis observed in these experiments were the result of the inability of the host to achieve effective levels of circulating antibody. This was not due to any significant degree of immunosuppression but rather to the continued rapid-replication rate of such strains of trypanosomes which constantly outpaced antibody production. In contrast, the strain of trypanosome which causes a more chronic infection, although initially having a similar replication rate, subsequently switched to a slower one and thereby allowed antibody to reach levels which permitted effective opsonization.

It is also possible, in sub-acute and chronic infections in which immunosuppression is well established, that the replication rate of particular variants might be equally important in determining the outcome of infection as recently suggested by Hudson & Terry (1979).

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