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The Nature of Immunosuppression in *Trypanosoma brucei* Infections in Mice

I. THE ROLE OF THE MACROPHAGE

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Summary. The functional integrity of the mononuclear phagocytic system (MPS) in mice infected with *Trypanosoma brucei* was investigated with regard to its possible significance in the aetiology of the immunosuppression characteristic of this disease.

In infected mice the spleens and lymph nodes were grossly enlarged, and on histological examination it was shown that the MPS of the liver, lymph nodes, spleen and bone marrow was markedly expanded. Macrophages presented an active appearance and often contained cellular debris. Clearance of intravenously injected sheep red blood cells (SRBC) was increased, in that 1 hour after injection, 2–6 times more ^{51}Cr-labelled SRBC had disappeared from the circulation of infected, compared to uninfected mice; this was due largely to an increased uptake by the expanded phagocytic system of the liver.

The intrinsic immunogenic potential of individual macrophages appeared to be unimpaired as judged by the ability of SRBC-containing macrophages from infected mice to elicit a response in syngeneic normal recipient mice.

It was concluded that the only evidence that immunosuppression might be associated with an altered activity of the MPS was an increased hepatic uptake of particulate antigen with a relative failure of splenic uptake. Together these might be responsible for a reduction in the concentration of antigen in the tissues of an enlarged spleen below the level necessary to initiate the formation of antibody.

INTRODUCTION

When mice are infected with *Trypanosoma brucei* there is a rapid depression of the immune response to a variety of other antigens including sheep red cells (Goodwin, 1970; Goodwin, Green, Guy and Voller, 1972; Murray, Urquhart, Murray and Jennings, 1973; Longstaffe, Freeman and Hudson, 1973). Associated with the development of the immunosuppression there is a marked cellular response in the lymphoid organs of the infected mice involving expansion of both the mononuclear phagocytic system, as defined by Furth, Cohn, Hirsch, Humphrey, Spector and Langevoort (1972), and the plasma cell series (Murray, Murray, Jennings, Fisher and Urquhart, 1974a).

The immunosuppression caused by *T. brucei* in mice is in many ways similar to that

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induced by another protozoan parasite, *Plasmodium berghei*. The defect caused by this latter parasite has been attributed to a 'disturbance of macrophage function produced by infection' (Greenwood, Playfair and Torrigiani, 1971), and Loose, Cook and Di Luzio (1973) have suggested that this dysfunction may be an impairment in macrophage antigen processing.

The results reported in this paper are concerned with possible alterations in the functional integrity of the expanded mononuclear phagocytic system of trypanosome-infected mice and their significance in the aetiology of immunosuppression. The role of other cellular components of the immune response is discussed separately (Murray, Jennings, Murray and Urquhart, 1974b).

MATERIALS AND METHODS

Animals

Female CFLP (Carworth Europe Ltd, Alconbury, Huntingdon) mice aged 6–10 weeks were used in all the experiments with the exception of those involving cell transfers where syngeneic female NIH mice (Carworth Europe Ltd) aged 6–10 weeks were used.

Inoculation and course of a typical infection

Each mouse was inoculated intraperitoneally with 1×10^4 organisms of a strain of *T. brucei* derived from the stabilate TREU 667. During the following 5 days, examination of wet blood smears from the tail vein showed no trypanosomes. Thereafter they were present, their numbers reaching a maximum by day 8 when about 200 trypanosomes per high power field were seen. After this point the number of trypanosomes declined rapidly but rose again within a few days. This cyclic variation in numbers continued throughout the course of the infection which persisted until the death of the host some 6–12 weeks after inoculation. During this period the mice remained lively and showed no signs of clinical disease other than moderate lymph node enlargement and abdominal distension associated with splenomegaly until a day or so before death when they became dull and lethargic; this terminal event was usually associated with a massive parasitaemia. The experiments described here, unless otherwise stated, were conducted on mice with an infection of 3 weeks duration.

Sheep erythrocytes

Sheep red blood cells (SRBC) in Alsever's solution (Wellcome Reagents Ltd, Beckenham, Kent) were washed three times in phosphate-buffered saline, pH 7·3 (PBS) before use in the cell transfer experiments. Fresh SRBC were used in the experiment where isotopic labelling with radioactive sodium chromate (^{51}Cr) was required. Mice were immunized by the intraperitoneal injection of 5×10^8 SRBC in 1 ml of PBS.

Haemagglutination

Six days after the SRBC immunization procedure, the mice were exsanguinated by cardiac puncture. The sera were inactivated by heating at 56° for 30 minutes. Duplicate doubling dilutions of the antisera were prepared in 25 μl of PBS in microtitre plates (Biocult Laboratories Ltd, Paisley). To each well was added 25 μl of a 2 per cent suspension of washed SRBC. The plates were covered and left overnight at room temperature before the haemagglutination titres were read. The antibody titres were read as being the highest dilution of antiserum giving complete agglutination.

^{51}Cr labelling

Fresh sheep blood, containing heparin as anticoagulant, was incubated with [^{51}Cr] sodium chromate for 30 minutes at 37°. This blood was then washed three times in PBS and the SRBC concentration adjusted after counting on a Coulter Counter (Coulter Electronics Ltd, Dunstable, Bedfordshire). 0·5 ml of the labelled cell suspension was injected intravenously. In the experiments where different doses of SRBC were required, unlabelled SRBC were added to the stock suspension containing the lowest numbers of ^{51}Cr-labelled SRBC; in this way the concentration was increased without any change in the total injected activity or the specific activity of each labelled cell.

Reticuloendothelial blockade

Carbon particules (C11/1431A Gunther Wagner, Pelikan Werke, Hannover) were suspended in a 1 per cent gelatin solution in distilled water (Souhami, 1972) and mice were blockaded by a single intravenous injection of this suspension at a dose of 16 mg carbon per 100 g body weight 6 hours before the injection of labelled SRBC.

Clearance and organ distribution of SRBC

Heparinized samples of blood were taken immediately before necropsy by cardiac puncture and centrifuged in microhaematocrit tubes. That portion of each tube containing the packed red cells was then weighed prior to and after expulsion of the contents into a counting vial containing 10 ml of diluted NaOH. All such samples were counted on an automatic well-type gamma scintillation counter (Nuclear Chicago, High Wycombe, Bucks) for a sufficient time to give a net count with less than 2 per cent standard error. All counts were expressed as counts/minute/mg packed red cells.

Each mouse was separated into liver, spleen and the remainder of the carcase and these were also assayed for ^{51}Cr activity.

Macrophage collection

Large numbers of macrophages were induced by the intraperitoneal (i.p.) injection of sterile thioglycollate medium (Difco Laboratories, West Molesey, Surrey) (Argyris, 1967) 6 days prior to collection. 5×10^8 washed SRBC in 1 ml of PBS were injected i.p. into the macrophage donor mice. Two hours later the peritoneal exudate (PE) cells were harvested by peritoneal lavage with 3 ml of Medium 199 (Wellcome Reagents Ltd, Beckenham, Kent) containing 5 units/ml heparin, 10 per cent foetal calf serum and 2 per cent Hepes buffer under sterile conditions. The PE cells were kept on ice at all times and cell counts were carried out in a haemocytometer using 2 per cent acetic acid with crystal violet as diluent. After the PE cells had been concentrated by centrifugation at 300 ***g*** for 5 minutes at 4°, the extracellular SRBC were lysed by the method of Morita and Perkins (1965) and the PE cells then washed in chilled Medium 199.

Macrophage culture

$1{\cdot}0 \times 10^7$ PE cells in 4 ml of medium were cultured in 65 mm tissue culture dishes for 40 minutes at 37°, after which the non-adherent cells were removed by shaking the culture dish and discarding the medium. The adherent cells were washed once in Medium 199 before being carefully scraped from the dish with a rubber-tipped spatula. The viability of the adherent cells was confirmed by their failure to stain with 0·5 per cent Trypan Blue; their phagocytic ability, as assessed by the ingestion of 0·1 per cent Neutral Red, was 85 per cent.

Macrophage transfer

$1{\cdot}0 \times 10^7$ adherent cells were transferred to each recipient by i.p. injection in 1 ml of Medium 199. Those mice receiving cells from *T. brucei*-infected donors were treated 1 day before the transfer was effected by the i.p. injection of 0·5 mg diminazene aceturate (Berenil, Farbwerke Hoechst, Frankfurt) in 0·1 ml of saline.

Histology

For histopathological examination, a group of mice were inoculated with *T. brucei* and killed in pairs at weekly intervals up to 10 weeks. Uninfected control mice were also killed at these intervals. Samples from all tissues and organs were fixed in Carnoy's fluid for 24–48 hours, dehydrated, and cleared in an alcohol–amyl acetate–chloroform series and embedded in paraffin wax. Sections were stained routinely with Haematoxylin and Eosin, and also with periodic acid–Schiff, Martius Scarlet Blue, Methyl Green–Pyronin and Perls' Prussian Blue (Pearse, 1968).

RESULTS

ONSET AND COURSE OF IMMUNOSUPPRESSION TO SRBC

The results presented in Table 1 show that the immunosuppressive effect was present within 1 week of infection and persisted throughout the course of the disease. However, when SRBC were given 2 or 4 days before the *T. brucei* infection, a normal haemagglutinin response was obtained.

TABLE 1

SUPPRESSION OF THE IMMUNE RESPONSE TO SRBC IN MICE INFECTED WITH *T. brucei* (TREU 667) AS SHOWN BY HAEMAGGLUTINATING TITRES OF SERA 6 DAYS AFTER IMMUNIZATION WITH 5×10^8 SRBC

Days between infection and immunization	Haemagglutinin titres*	
	Infected	Normal
−4	7·4 ± 0·5	7·2 ± 0·6
−2	7·3 ± 0·5	—
+3	6·3 ± 0·6	8·5 ± 0·4
+7	Negative	8·2 ± 0·5
+21	Negative	7·6 ± 0·4
+70	Negative	6·6 ± 0·5

* Each value is the mean $\log_2 \pm$ s.e. of ten mice. The sera of infected and normal mice, not immunized with SRBC, were negative.

CHANGES IN THE MONONUCLEAR PHAGOCYTIC SYSTEM (MPS)

Within a few days of inoculation, and persisting throughout the infection, there was a marked increase in numbers and activity of the cells of the MPS of the liver, lymph nodes, spleen, bone marrow and also of non-fixed macrophages of all tissues. In the liver, the Kuppfer cells were prominent, increased in number and were often found in mitosis. In addition, non-fixed macrophages and monocytes were common in the blood vessels and tissues of the liver. Lymph node sinuses were packed with macrophages (Fig. 1) and these cells were also prominent in the medullary cords and the paracortical area; in the spleen, macrophages were numerous. In infected mice, the spleens were grossly enlarged. Thus, 3

weeks after infection, their mean weight was 1·44 g compared to a normal spleen weight of 0·13 g. In cases of longer duration they frequently weighed as much as thirty times normal. This was due not only to engorgement with erythrocytes, but also to a marked increase in nucleated cells including macrophages; these cells were most commonly found in the sinuses and cords of the red pulp and in the marginal zone surrounding the white pulp. Macrophages were also numerous in bone marrow and throughout the organs and tissues of the body, especially where trypanosomes were located. In all such areas macrophages had an active appearance with abundant vacuolated cytoplasm which often contained debris; many contained chromatin-like particles, possibly ingested trypanosomes, while in the spleen they were frequently packed with erythrocytes (Fig. 2).

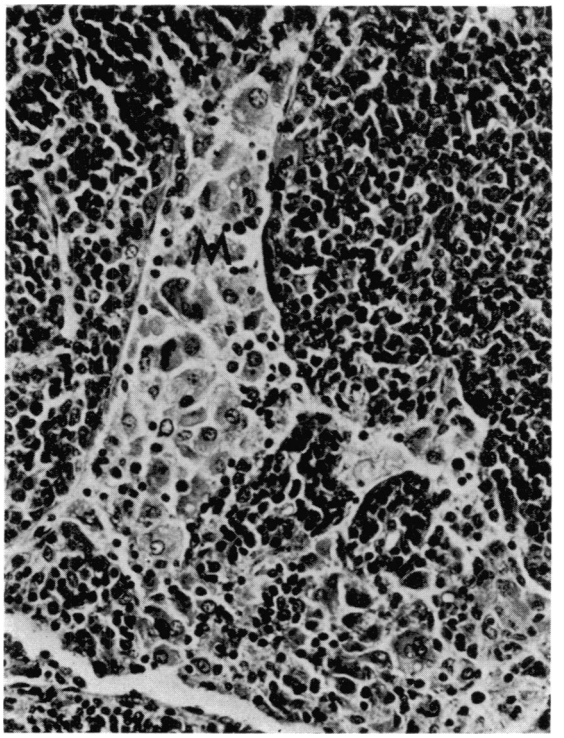

FIG. 1. Lymph node medulla in mouse 21 days after inoculation with *T. brucei*. The medullary sinus (M) and medullary cord contain numerous macrophages. Note the plasma cells in the medullary cords. (Haematoxylin and Eosin, magnification ×270).

CLEARANCE OF ^{51}Cr-LABELLED SRBC FROM THE CIRCULATION

SRBC are removed rapidly from the circulation of the trypanosome-infected mice (Table 2) indicating an expanded MPS and possibly an enhanced ability for erythrophagocytosis.

THE HEPATIC AND SPLENIC DISTRIBUTION OF ^{51}Cr-LABELLED SRBC

Table 3 shows the distribution of radioactivity in the livers and spleens of infected and normal mice 24 hours after the intravenous injection of ^{51}Cr-labelled SRBC. From this it

is apparent that the rapid removal of red cells from the circulation of the former appeared to be largely due to the increased phagocytic ability of the liver. In contrast, the number of SRBC which localized in the spleen was the same in both normal and infected mice.

This was somewhat surprising in view of the marked splenomegaly and associated macrophage expansion and raised the possibility that the concentration of antigen in the spleen might be so low as to fail to stimulate production of antibody. In an effort to increase the amount of SRBC deposited in the spleen a prior injection of carbon was given before the administration of ^{51}Cr-labelled SRBC. In normal mice Souhami (1972) has shown that

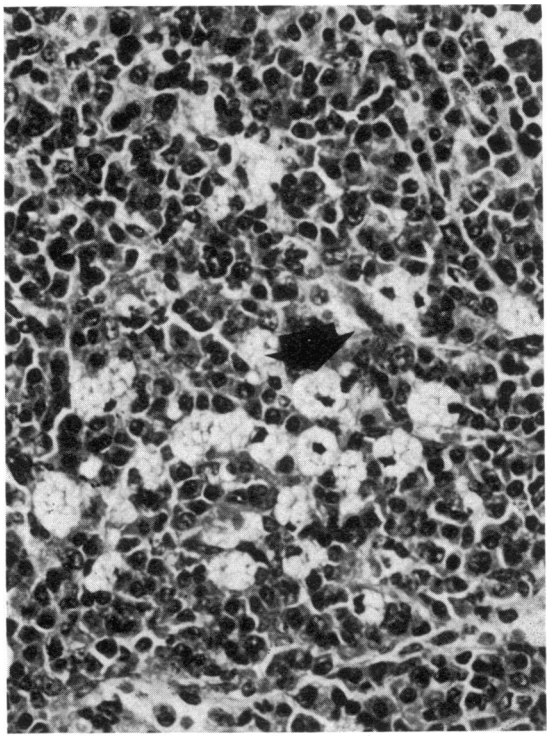

FIG. 2. Splenic red pulp in mouse 28 days after inoculation with *T. brucei*. Macrophages are packed with red blood cells (arrow) and are surrounded by numerous plasma cells. (Haematoxylin and Eosin, magnification ×315).

this produces a blockade of the hepatic phagocytes and a diversion of SRBC to the spleen. While this was also observed in our normal mice (Table 4), it failed to occur in the trypanosome-infected mice, the expanded phagocytic systems of their livers apparently coping readily with both types of particulate material.

MACROPHAGE TRANSFER EXPERIMENTS

In addition to phagocytic ability it is likely that the presentation of processed antigen in an immunogenic configuration on the macrophage plasma membrane is a vital component for macrophage participation in the immune response (Unanue and Cerottini, 1970). To study this aspect, macrophage transfer experiments were performed as described below. Macrophages, allowed to ingest SRBC *in vitro*, were transferred by intraperitoneal

injection to syngeneic recipient mice. Both infected and normal mice were used as macrophage donors and recipients. Six days after transfer, sera of these recipients was assayed for haemagglutinating antibody to SRBC. The results are shown in Table 5 and demonstrate that, while macrophages containing SRBC from both normal and infected mice initiated an immune response when injected into normal mice, they failed to elicit a significant response in the presence of *T. brucei* infection in the recipients.

TABLE 2

THE ACTIVITY REMAINING IN THE CIRCULATION OF MICE 1 HOUR AFTER THE INJECTION OF ^{51}Cr-LABELLED SRBC

Number of injected SRBC ($\times 10^9$)	Activity (c.p.m./mg erythrocytes)*	
	Infected	Normal
5·0	1108±262	2069±162
2·0	330±139	733±131
0·9	42±39	275±34

* Each value is the mean±s.e. of eight to ten mice.

TABLE 3

THE ACTIVITY IN THE LIVERS AND SPLEENS OF MICE 24 HOURS AFTER THE INTRAVENOUS INJECTION OF ^{51}Cr-LABELLED SRBC*

Number of injected SRBC	Infected		Normal	
	Spleen	Liver	Spleen	Liver
2×10^9	15·8±2·2	62·8±8·9	18·9±2·2	16·1±0·7
2×10^8	3·0±0·8	91·3±1·5	4·8±1·2	72·0±3·7
2×10^7	2·8±0·7	89·1±3·0	1·2±0·2	90·7±1·1

* Each value is the mean±s.e. of three to eight mice expressed as a percentage of the total body activity at necropsy.

TABLE 4

THE EFFECT OF PRIOR CARBON BLOCKADE ON THE ACTIVITY IN THE LIVERS AND SPLEENS OF MICE 2 AND 24 HOURS AFTER THE INJECTION OF 2×10^8 ^{51}Cr-LABELLED SRBC*

		Infected		Normal	
		No carbon	With carbon	No carbon	With carbon
2 hours	Spleen	2·3±0·6	3·9±0·6	3·0±0·8	18·7±3·3
	Liver	88·6±1·2	88·9±1·2	85·7±1·5	59·9±4·3
24 hours	Spleen	3·0±0·8	1·7±0·4	4·8±1·2	24·7±2·3
	Liver	91·3±1·5	93·7±1·0	72·0±3·7	46·8±3·6

* Each value is the mean±s.e. of three to eight mice expressed as a percentage of the total body activity.

DISCUSSION

The haemagglutination results reported in the first part of the paper confirm that *T. brucei* infection of rodents is associated with a profound degree of immunosuppression to SRBC, which becomes apparent between days 3 and 7 after infection and persists for

up to 10 weeks at least. In the following paper (Murray *et al.*, 1974b) similar results were obtained using a plaque-forming cell technique where, even as late as 8 days after SRBC immunization, the spleen cells of infected mice showed no evidence of specific antibody production. Thus the phenomenon appears to be one of immunosuppression rather than merely the delayed appearance of circulating antibody.

Since a defect in the macrophages may be responsible for a failure of the induction phase of the immune response, the second part of the paper was therefore concerned with an examination of the functional integrity of the mononuclear phagocytic system in infected mice as determined in three ways. First, a histopathological appreciation of macrophage numbers and morphology in the organs of infected mice; secondly, quantitative studies of their ability to remove injected SRBC from the circulation and their subsequent distribution in the liver and spleen; finally, the ability of these cells to 'process and transfer' antigenic information to the antibody-forming apparatus.

TABLE 5

THE IMMUNOGENICITY OF SRBC INGESTED BY MACROPHAGES AND TRANSFERRED TO SYNGENEIC RECIPIENTS

Presentation of SRBC	Recipient mice		Mean $\log_2$ agglutination titre ± s.e.	Significance
In macrophages from normal mice	Normal	(6)*	3·3 ± 0·3	$P<0·001$
	Infected	(5)	0·6 ± 0·4	
In macrophages from infected mice	Normal	(4)	3·3 ± 0·5	$P<0·05$
	Infected	(3)	1·3 ± 0·3	
In PBS	Normal	(6)	5·7 ± 0·9	$P<0·001$
	Infected	(5)	1·0 ± 0·3	
Nil	Normal	(7)	Negative	—
	Infected	(5)	0·6 ± 0·4	

* Values in parentheses are the number of mice in each group.

Histological examination showed a marked expansion of the MPS of the liver, spleen and bone marrow. Not only were the cells increased in number, but also their morphological appearance indicated that they were active with abundant vacuolated cytoplasm often containing cellular debris. The cause of the expanded MPS is unknown but it may be that a mechanism similar to that proposed for tropical splenomegaly syndrome in man is operative. In this it has been postulated that the expanded MPS particularly in the liver and spleen is produced by high levels of circulating macromolecular immune complexes (Ziegler, 1973).

In view of the vastly expanded MPS, it is perhaps not surprising that injected SRBC were removed very rapidly from the circulation. Thus, 1 hour after injection, 2–6 times more ^{51}Cr-labelled SRBC had disappeared from the circulation in infected, compared to uninfected, mice. This rapid clearance of SRBC raises the question of whether any of the immunosuppressive effects are attributable to the expanded MPS *per se*. Warr and Šljivić (1973) and Šljivić and Warr (1973) have shown that stimulation of the reticuloendothelial system by stilboestrol leads to an increased hepatic uptake of intravenously injected particulate materials such as SRBC, apparently the result of specific activation of the Kuppfer cells in the liver. This is associated with reduction in the splenic localization of SRBC and a consequently diminished antibody response.

Our attempts to investigate the possibility that the expanded hepatic phagocytic system

in trypanosome-infected mice is responsible for immunosuppression, have yielded some equivocal results. Thus the percentage uptake of ^{51}Cr-labelled SRBC in the spleen and liver 2 hours after injection was 3 per cent and 86 per cent respectively in normal mice and 2 per cent and 89 per cent in infected mice. This indicated that a similar quantity of antigen was localized in the spleens in both cases. However, in view of the vastly increased splenic size in infected mice, i.e. up to thirty times normal, it is possible that the concentration of antigen may have been insufficient to initiate the production of antibody.

In an attempt to increase the quantity of antigen reaching the spleen colloidal carbon was given intravenously to mice prior to the injection of ^{51}Cr-labelled SRBC. Souhami (1972) has shown that this technique results in the reticuloendothelial blockade of hepatic phagocytes and a consequent increase in splenic uptake of antigen. While this occurred in our control mice (the splenic uptake of ^{51}Cr-labelled SRBC increased from 3–19 per cent of the injected dose), the trypanosome-infected mice showed virtually the same relative distribution of ^{51}Cr-labelled SRBC both with and without the injection of carbon. It would thus appear that the hepatic phagocytic system in mice infected with trypanosomes was so expanded or activated that it readily coped with both carbon and red cells. This failure to obtain even a relative increase in splenic concentration of SRBC might be due to the fact that splenic engorgement leads to some degree of haemostasis and a consequently slow passage of the antigen through the spleen. Thus, over a given period of time it is likely that the liver would retain a disproportionate amount of antigen. Whatever the reason, the proportion of SRBC retained by the spleen in trypanosome-infected mice was nevertheless similar to that of normal mice and was associated with complete failure of antibody production in the former. Clearly the possibility that expansion of the MPS in mice with trypanosomiasis is responsible for the immunosuppression requires further investigation.

Apart from the rate of uptake and final distribution of injected antigen in trypanosome-infected mice, one other aspect of macrophage function was examined, i.e. the ability of the macrophage to co-operate with other cell types in the immune response by the suitable processing and presentation of SRBC antigens. This particular role was examined by the transfer of peritoneal macrophages containing SRBC from trypanosome-infected mice to the peritoneal cavities of normal mice. The results show that such macrophages were perfectly capable of initiating a significant antibody response. In contrast, when the same number of normal macrophages containing SRBC were transferred to trypanosome-infected mice, no antibody was produced. From this experiment it is apparent that the intrinsic immunogenic potential of the macrophage in infected mice is unimpaired.

In conclusion, the only evidence presented here which might indicate that the MPS is at least a contributory factor in the development of the immunosuppressive phenomena associated with trypanosomiasis was the finding that the amount of injected SRBC which localized in the spleen was not increased despite an increased rate of removal of SRBC from the circulation and gross splenomegaly. Even a degree of hepatic blockade, normally sufficient to produce increased splenic localization of subsequently injected SRBC, failed to increase uptake by this organ in infected mice.

It appears possible therefore that these two factors, i.e. increased hepatic uptake of particulate antigen and a possible selective failure of splenic uptake, might be responsible for a reduction in the concentration of antigen in the tissues of an enlarged spleen below the level necessary to initiate the formation of antibody. It is likely, however, that other factors, as well as the macrophage, are associated with immunosuppression in trypanosomiasis and some aspects of these are discussed in the next paper (Murray *et al.*, 1974b).

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