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African trypanosomiasis alters prostaglandin production by murine peritoneal macrophages

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SUMMARY

Many factors contribute to the severe immunosuppression associated with African trypanosomiasis. Macrophages have been shown to be important target cells which after uptake of parasites, mediate immune dysfunction *in vivo*. We observed that infection of mice with *Trypanosome brucei brucei* (clone NIM 6) induces profound changes in arachidonic acid metabolism and prostaglandin (PG) secretion by macrophages. Normal macrophages release more PGI_2 than PGE_2 and production of both these prostaglandins is stimulated equally by endotoxin (LPS). Macrophages taken from NIM 6 infected mice at the peak of the first parasitaemia, release increased amounts of PGE_2 and are hyperresponsive to LPS stimulation, while PGI_2 secretion remains normal. As the infection progresses, there is a striking decrease in both basal PGE_2 and PGI_2 secretion and the ability of macrophages to respond to LPS. By the third week of infection, shortly before death, peritoneal macrophages resemble thioglycollate elicited macrophages in their inability to be stimulated by LPS to synthesize prostaglandins. Infection with a more virulent clone of *T. brucei* (NIM9) results in suppression of both PGE_2 and PGI_2 release by day 9 of infection. The increased production of PGE_2 by macrophages during the height of infection is likely to contribute to the general immunosuppression associated with African trypanosomiasis.

Keywords macrophage activation African trypanosomiasis immunosuppression prostaglandin E_2

INTRODUCTION

Natural and experimental infections with *Trypanosoma brucei* severely inhibit immune responses (Goodwin *et al.*, 1972). In a mouse model, infection or even injection of trypanosome membranes results in proliferation of B and T cells followed by loss of immune responsiveness to heterologous antigens (e.g. Hudson *et al.*, 1976; Mansfield, 1978), and most importantly, to parasite antigens themselves (e.g. Sacks & Askonas, 1980; Gasbarre, Hug & Louis, 1981). The severity of these alteration increases during infection and also varies with the virulence of the infecting strain of *T. brucei* (Sacks *et al.*, 1980). In view of antigenic variation by the parasite, the immunosuppression is likely to contribute to the fatal outcome of this infection, and the increased susceptibility of hosts with trypanosomiasis to secondary infections.

The cellular mechanisms responsible for immunosuppression in African trypanosomiasis have

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been studied most thoroughly in inbred mice. In the course of infection, suppressive T cells and macrophages are generated (Corsini *et al.*, 1977; Jayawardena, Waksman & Eardley, 1978; Pearson *et al.*, 1978; Wellhausen & Mansfield, 1979). B cell abnormalities develop also in congenitally athymic mice after infection with *T. brucei* indicating some T-independent suppressive pathways (e.g. Clayton, Ogilvie & Askonas, 1979). One of the direct target cells for parasite action is the macrophage since small numbers of macrophages, after ingestion of trypomastigotes, can mediate immunosuppression (Grosskinsky & Askonas, 1981), possibly by releasing suppressive 'factors'. Of all the products released by macrophages (Nathan, Murray & Cohn, 1980), prostaglandin E_2 (PGE_2) seemed a likely candidate since it inhibits B and T lymphocyte functions (e.g. Goodwin & Webb, 1980). We therefore examined the effect of trypanosomiasis on the production by peritoneal macrophages of prostaglandins (PG), namely PGE_2, 6-keto-$PGF_{1\alpha}$, the stable hydrolysis product of prostacyclin (PGI_2), and thromboxane B_2 (TXB_2), the stable product of thromboxane A_2 (TXA_2). We report that at the time of strong immunosuppression, unstimulated NIM 6 macrophages from infected mice produce normal amounts of PGI_2 but supranormal amounts of PGE_2. Furthermore, when these macrophages are incubated with endotoxin (LPS), they are stimulated to synthesise more PGE_2 but not PGI_2. As a result, there is a reversal of the normal ratio of PGI_2/PGE_2 released from LPS stimulated macrophages.

MATERIALS AND METHODS

Mice. (CBA × C57B1/6)F_1 or (CBA × BALB/c)F_1, female mice were bred at N.I.M.R. under SPF conditions and used at 4–6 months of age.

Trypanosome clones and infections. T. brucei clone NIM 6 was derived from primary isolate Serengeti/66/SVRP/42 (Sacks *et al.*, 1980). Infection of (CBA × BALB/c)F_1 mice resulted in a first wave of parasitaemia (days 4–9), followed by a second wave of variant parasites and death after 25–28 days. *T. brucei* clone NIM 9 caused a progressive infection that leads to death within 10 days (Sacks *et al.*, 1980). Mice were infected with 400 parasites i.p. and parasitaemia was estimated by examining wet blood films under 400-fold magnification.

Macrophages and incubation conditions. The peritoneal cells of three to five mice were harvested with 10 ml of iced PBS, without Ca or Mg, containing 2·5 u/ml of sodium heparin. After centrifugation at 240*g* for 10 min at 4°C, the cells were resuspended in RPMI 1640 plus 1% FCS (heat-inactivated) with added penicillin and streptomycin. Viable cells, ($2–3 \times 10^6$ in 1·5 ml) were added to 30 mm plastic Petri dishes (NUNC) and incubated at 37°C in a 6% CO_2 atmosphere for 2 h. Non-adherent cells including trypanosomes were removed by washing three times with Hanks' balanced salt solution (HBSS) and then 2 ml of RPMI + 1% FCS were added, with or without 5 μg/ml LPS (*E. coli* 055:B5, DIFCO). Each assay was performed in duplicate. Higher percentages of FCS were found to inhibit the production of PGE_2 and PGI_2 by stimulated cells as has been observed by others (Scott *et al.*, 1980).

Adherent cells were at last 95% macrophages as judged by typical spreading morphology using phase contrast microscopy, and phagocytosis of latex beads. Less than 1% of adherent cells were stained by FITC conjugated IgM monoclonal anti-Thy-1 (NIMR 1, a gift of Ann Chayen) and no free trypanosomes remained. Because the percentage of adherent cells differed from group to group, we determined the number of adherent cells by incubating the monolayer at 37°C for 1 h with 1 ml of 0·1 M citric acid containing 0·1% crystal violet (wt/vol.) (Sanford *et al.*, 1950). The cell monolayer was washed vigorously with citric acid before and after incubation. The number of released nuclei were counted from duplicate dishes using a haemocytometer and less than 10% of cell nuclei remained attached.

Radioimmunoassay (RIA). PGE_2, 6-keto-$PGF_{1\alpha}$ and TXB_2 were measured by RIA as previously described (Salmon, 1978). Briefly, rabbit antiserum to each compound was incubated with test samples and tritiated standards. Free radioactive material was removed with dextran coated charcoal and residual bound radioactivity measured. The assays for PGE_2 and TXB_2 are specific; there is a 5% cross-reactivity of PGE_2 with the anti-6-keto-$PGF_{1\alpha}$ serum (Salmon, 1978). We did not adjust the 6-keto-$PGF_{1\alpha}$ results to compensate for this cross-reactivity. Because the

RPMI-1% FCS contained 0·75 ng/ml immunoreactive TXB_2, that amount was substracted from all results. All assays were done in duplicate.

Labelling of macrophages with arachidonic acid, lipid extraction and analysis. ^{14}C-arachidonic acid in toluene (176 μCi/mg, Radiochemical Centre, Amersham). The toluene was evaporated using a nitrogen stream and the arachidonic acid was then redissolved in absolute ethanol. We added 5 μl containing 0·250 μCi to 10^6 adherent macrophages in 2 ml of RPMI-1% FCS. Control macrophages were incubated with 5 μl of ethanol and neither their viability nor their production of PGE_2 and 6-keto-$PGF_{1\alpha}$ was affected. The adherent cells were incubated with the radiolabelled arachidonic acid for 3 h and washed three times with HBSS plus 1% bovine serum albumin followed by one wash with RPMI + 1% FCS. After re-incubating with or without LPS for 2 h, the supernatant was removed and stored as above.

Samples were extracted with 2 vols of cold acetone; the precipitate was re-extracted with acetone and the extracts were combined. Neutral lipids were removed from the aqueous acetone with n-hexane. The aqueous acetone was adjusted to pH 4 with formic acid and then was extracted with 2 vols of chloroform. After evaporation under nitrogen, the extract was dissolved in 50 μl chloroform: methanol (2:1) and applied quantitatively to a Whatman LKD thin layer chromatography plate. Standards were applied to the same plate which was developed with the organic phase of a mixture of ethyl acetate: trimethylpentane: acetic acid: water (110:50:20:100) (Salzman, Salmon & Moncada, 1980). Plates were dried and placed on X-ray film (Kodak) for autoradiography.

RESULTS

PG products of macrophages

To ascertain which PG products were secreted, we analysed the radiolabelled products released by macrophages pre-labelled with ^{14}C-arachidonic acid. Fig. 1 shows that normal macrophages incubated with or without LPS produce primarily PGE_2 and 6-keto-$PGF_{1\alpha}$. A different pattern of PG release could be discerned in the supernatant of macrophages from mice 12 days after NIM 6 infection (NIM 6 macrophages). These cells released very little PGE_2 or 6-keto-$PGF_{1\alpha}$ and much less ^{14}C-arachidonic acid. Stimulation of the NIM 6 macrophages with LPS increased particularly the formation of PGE_2 and the release of unaltered arachidonic acid. PG production was totally inhibited by indomethacin as expected. Almost no detectable TXB_2 or $PGF_{2\alpha}$ was produced by either group of macrophages, so in most subsequent experiments we only determined concentrations of 6-keto-$PGF_{1\alpha}$ and PGE_2.

Quantitation of PG release during infection

PG levels were analyzed quantitatively by RIA. Peritoneal macrophages (10^6) from normal F_1 mice produced about 20 ng of 6-keto-PGF_1 (mean of five experiments) and about half that amount of PGE_2 in 2 h (Fig. 2). LPS stimulation more than doubled this rate of PG production but the ratio of 6-keto-$PGF_{1\alpha}$ to PGE_2 remained greater than 2. In contrast, NIM 6 macrophages (day 12) released less than 5 ng of both PGE_2 and 6-keto-$PGF_{1\alpha}$ in 2 h, but after LPS stimulation, PGE_2 levels increased dramatically (10-fold) while 6-keto-$PGF_{2\alpha}$ increased only three-fold (Fig. 2). As a result, the ratio of 6-keto-$PGF_{1\alpha}$ to PGE_2 was 0·3 for NIM 6 macrophages. The preferential release of PGE_2 was greatly accentuated by prolonging the incubation with LPS to 8 or 24 h (Fig. 3); during a 24 h incubation NIM 6 macrophages produced three times as much PGE_2 as normal macrophages.

When peritoneal macrophages were analysed at several intervals after infection, 6-keto-$PGF_{1\alpha}$ synthesis was if anything less than normal throughout the infection except on day 6. After that, inhibition of 6-keto-$PGF_{1\alpha}$ synthesis was progressive so that by day 19, cells released less than 1% of normal amounts (Fig. 4). PGE_2 synthesis was affected in a different manner. Coincident with the first peak of parasitaemia there was a six-fold increase in PGE_2 synthesis by LPS stimulated cells and a four-fold increase by unstimulated cells over normal levels. The synthesis of PGE_2 then declined slowly so that by day 19, levels were only 20% of normal. TXB_2 synthesis by unstimulated normal and NIM 6 macrophages was too low to be detected over the background of TXB_2 in the FCS. Although LPS stimulated some synthesis of TXB_2, this accounted for less than 10% of the total PG production (not illustrated).

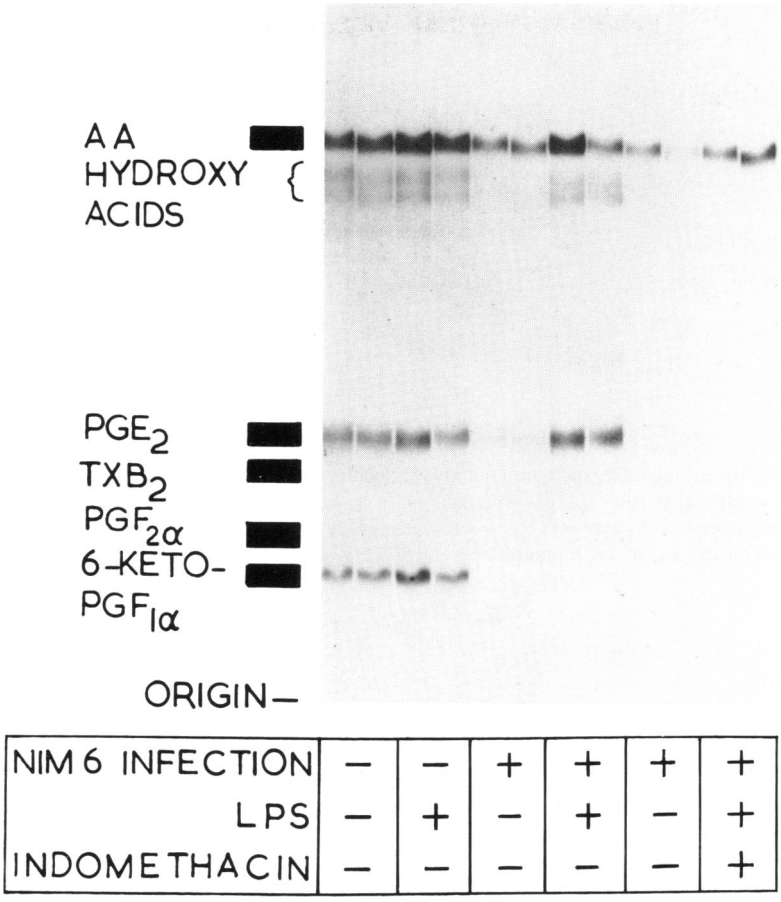

Fig. 1. Conversion of ^{14}C-arachidonic acid by peritoneal macrophages. Macrophages were pooled from four normal infected mice (day 12 of NIM 6 infection). After labelling with arachidonic acid, macrophage cultures were incubated for 2 h in duplicate and the supernatant samples analysed by thin layer chromatography and autoradiography (see Materials and Methods). Solid bars indicate the location of authentic standard compounds chromatographed along side the macrophage supernatants. Indomethacin (5×10^{-6}M) or LPS (5 μg/ml) were added to the incubation as indicated. AA represents arachidonic acid.

We infected mice with virulent clone, NIM 9, which causes a progressive infection that kills mice within 9–10 days. Macrophages obtained on day 9 at a time of heavy parasitaemia ($> 5 \times 10^8$/ml) showed a strikingly reduced PG release and could not be stimulated by LPS (Table 1). These alterations in arachidonic acid metabolism are similar to the changes produced by thioglycollate.

DISCUSSION

There is a general agreement that normal macrophages can be stimulated by phagocytosis, antigen–antibody complexes and LPS to convert arachidonic acid to prostaglandins (Bonney *et al.*, 1979; Kurland & Bockman 1978). Using thin layer radiochromatography and radioimmunoassay, we found that resident mouse peritoneal macrophages secreted more PGI_2 (measured as the stable metabolite, 6-keto-$PGF_{1\alpha}$) than PGE_2, and that LPS increased the secretion of both fatty acids without altering the ratio of 6-keto-$PGF_{1\alpha}/PGE_2$. This contrasts results by Humes *et al.* (1977) but

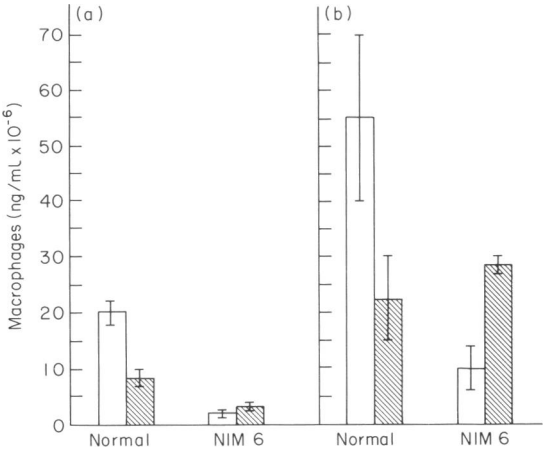

Fig. 2. Change in prostaglandin production following infection with clone NIM 6. Peritoneal macrophages were obtained from normal (CBA × BALB/c) F_1 mice or from mice 12 days after infection with clone NIM 6. Incubation of adherent cells was with (b) or without (a) 5 μg LPS/ml for 2 h. □ = 6-keto-$PGF_{1\alpha}$; ▩ = PGE_2. Values are the mean ± s.e. of five separate experiments.

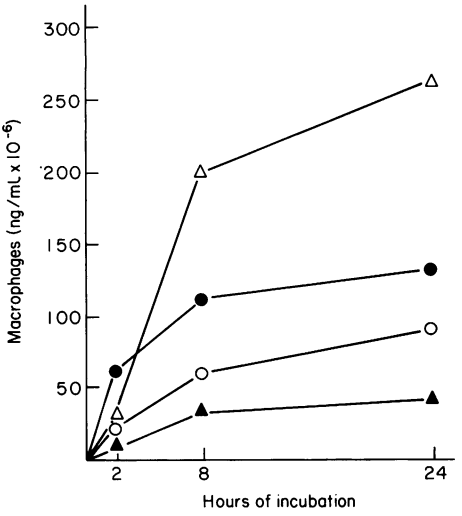

Fig. 3. Kinetics of production of 6-keto-$PGF_{1\alpha}$ and PGE_2 by adherent peritoneal macrophages from normal or NIM 6 infected mice (day 12 of infection). LPS (5 μg/ml) was present throughout the incubation. Each point is the mean of duplicate cultures of macrophages obtained from six normal or six infected mice. PGE_2, NIM 6 macrophages (△——△); PGE_2, normal macrophages (▲——▲); 6-keto-$PGF_{1\alpha}$, NIM 6 macrophage (○——○), 6-keto-$PGF_{1\alpha}$, normal macrophages (●——●).

others also have found that macrophages from healthy mice secrete more 6-keto-$PGF_{1\alpha}$ than PGE_2 (e.g. Rossello *et al.*, 1981, Scott *et al.*, 1982).

The major finding of this study was that infection with *T. brucei brucei* resulted in profound changes in the arachidonic acid metabolism by peritoneal macrophages. Coincident with the first wave of parasitaemia there was an increase in basal and LPS stimulated synthesis of PGE_2; this was followed by a progressive decrease in PGE_2 synthesis, which could not be enhanced by LPS late in infection. In contrast, infection only led to progressive suppression of the ability of macrophages to synthesise PGI_2. The level of TXB_2 synthesis was not altered by infection so that by day 18 TXB_2 represented nearly 50% of total PG synthesis (data not shown).

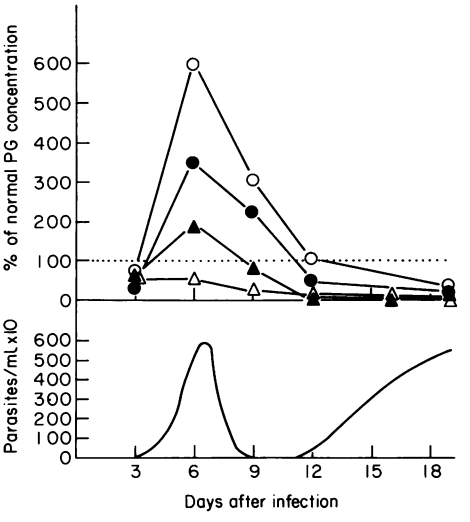

Fig. 4. (a) Production of 6-keto-$PGF_{1\alpha}$ and PGE_2 during a 2 h incubation of peritoneal macrophages obtained from (CBA × BALB/c) F_1 mice at various stages after infection with clone NIM 6. The results are expressed as a percentage of the secretion rates of normal peritoneal macrophages incubated under the same conditions. PGE_2, unstimulated ●——●; PGE_2, LPS stimulated ○——○; 6-keto-$PGF_{1\alpha}$, unstimulated ▲——▲; 6-keto-$PGF_{1\alpha}$, LPS stimulated △——△. Each point represents the mean of two separate experiments. The course of the parasitaemia in these mice is plotted in (b) on the same time scale.

Table 1. Inhibition of PG synthesis by macrophages from mice infected with *T. brucei* clone NIM 9

Macrophages	LPS (5 μg/ml)	6-keto-$PGF_{1\alpha}$ (ng/ml × 10^{-6})	PGE_2 macrophages
Normal	−	23·0	10·0
	+	70·0	15·0
NIM 9 infection*	−	1·5	1·0
	+	2·5	2·0
Thioglycollate† induced	−	2·5	1·0
	+	3·5	3·0

Macrophages from (C57Bl/6xCBA) F_1 mice were incubated for 2 h at 37°C in RPMI-1% FCS.

* Day 9 of infection.

† Macrophages were obtained 72 h after i.p. injection of 1·5 ml thioglycollate broth.

Macrophages can be induced *in vitro* by many inflammatory and microbial products to synthesize prostanoids (reviewed by Gemsa *et al.*, 1982). The increase in PGE_2 synthesis by macrophages during the first wave of parasitaemai may follow the uptake of some parasites in the presence of early antibody, or interaction with parasite product(s). Later in infection (days 12–18), after the macrophages have ingested a very large parasite load, the phagocytes resembled thioglycollate elicited (Humes *et al.*, 1977) or *C. parvum* elicited macrophages (Scott *et al.*, 1982) in that prostanoid synthesis was inhibited. The changing pattern of PG synthesis may reflect different stages of macrophage activation by increasing parasite loads. Macrophages also synthesize leukotrienes (see Samuelsson, 1981) but the activities of this class of compounds on the immune response are, as yet, ill-defined. There is some evidence that leukotriene LB4 induces the generation

of suppressive cells (Goodwin & Atluru, 1984) but as the leukotrienes were not determined in the present study we cannot speculate as to their role in trypanosomiasis.

The experiments with macrophages pre-labelled with ^{14}C-arachidonic acid aid the interpretation of our data. The release of ^{14}C-arachidonic acid from unstimulated NIM 6 macrophages (day 12) was less than normal. Since exogenous labelled arachidonate is incorporated into membrane phospholipid by macrophages (Scott *et al.*, 1980), failure to release the labelled fatty acid indicates inhibition of phospholipase activity. However, LPS stimulation of these cells resulted in normal release of arachidonic acid; implying that phospholipase was not irreversibly inhibited, or that a second phospholipase pool was activated by LPS as suggested by Hsueh *et al.* (1981). LPS stimulated macrophages from NIM 6 infected mice also were able to convert a high percentage of the released arachidonate to PGE_2 and this indicated that the cyclo-oxygenase enzyme was functional. The increased release of PGE_2 but lowered synthesis of 6-keto-$PGF_{1\alpha}$ in cells from infected mice implies an inhibition of prostacyclin synthetase. The latter inhibition could be due to the increased formation of oxygen radicals by infected cells; such oxidants are known to block prostacyclin synthesis (Moncada *et al.*, 1976; Salmon *et al.*,1978; Ham *et al.*, 1979).

Prostaglandin synthesis by freshly harvested macrophages was mainly assessed after 2 h *in vitro*. Therefore, the measured secretory rates should reflect the behaviour of these cells *in vivo*. NIM 6 infection causes many alterations in macrophage function (Grosskinsky *et al.*, 1983) and surface receptors (Fierer & Askonas, 1982) with an inverse relation between PGE_2 production and expression of Fc1, C3bi, and mannose receptors. Since PGE_2 inhibits both T and B cell functions (Goodwin & Webb, 1980), and down regulates Ia on macrophages (Snyder, Beller & Unanue, 1982) increased synthesis of PGE_2 in *T. brucei* NIM 6 infection may play a role in the macrophage mediated immunosuppression. As there is almost no information about the effects of PGI_2 on lymphocytes, we cannot comment of the effect that decreased PGI_2 may have on the immune response. It is probable that prostaglandins from macrophages also mediate some of the inflammatory responses which are associated with *T. brucei* infections, as has been reported in other inflammatory exudates (Stenson & Parker, 1980). There is evidence that bradykinin mediates some of the tissue damage that occurs in African trypanosomiasis (Boreham, 1970) and vasoactive prostaglandins are known to act synergistically with bradykinin leading to increased vascular permeability and oedema formation (Moncada, Ferreira & Vane, 1973), a complication of *T. brucei* infection (Goodwin, 1970).

Little is known about the effects of other infections on prostaglandin production by macrophages. Cahill & Hopper (1982) found that infection with an avirulent strain of *Salmonella enteritis* results in suppression of PGE_2 production by peritoneal macrophages and there was an inverse correlation between the number of intracellular bacterial and PGE_2 secretion. Salmonella are facultative intracellular pathogens whereas *T. brucei is an* extracellular parasite. However, once antibodies are formed, macrophages remove an enormous mass of trypomastigotes from the circulation so they may resemble macrophages that have ingested live bacteria.

It is clear that African trypanosomiasis results in many alterations in the immune system (see reviews by Mansfield, 1978; Bancroft & Askonas, 1982; Roelants & Pinder, 1984), and changes in PG synthesis during the course of infection are only one of many factors likely to contribute to the immune dysfunction.

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