The relationships between endotoxins, nitric oxide and inflammatory cytokines in blood and intestinal tissues in experimental *Trypanosoma brucei brucei* infections

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SUMMARY

Increased levels of circulating endotoxins are a feature of both human and experimental African trypanosomiasis. Studies with rats and mice have shown that these may originate from intestinal damage with altered permeability of the gut epithelium. Endotoxins are potent immunomodulatory substances which can initiate the production of a range of cytokines and mediators from different cell types. In rats infected with *T.b. brucei* we have examined possible associations of the endotoxin increases with increases in levels of TNF-α, IL-1β, IL-6, IFN-γ and nitric oxide (NO). Significant increases in each substance occurred at days 21 and 33 post-infection (p.i.). The increases in cytokines were highly correlated with the endotoxin levels (e.g. at day 21 p.i. the correlation–regression values were as follows: TNF-α, $r = 0{\cdot}9$, $P < 0{\cdot}01$; IL-1β, $r = 0{\cdot}83$, $P < 0{\cdot}01$; IL-6, $r = 0{\cdot}9$, $P < 0{\cdot}01$; IFN-γ, $r = 0{\cdot}7$, $P < 0{\cdot}01$). There were also strong correlations between the increased levels of several individual cytokines. Biopsies of chopped sections of small intestine tissues of rats showed a parallel production of cytokines, again with significant correlations with the circulating endotoxins. The production of NO and cytokines by the intestine may be associated with the increased transepithelial permeability which occurs during the infection.

Key words: experimental African trypanosomiasis, pathogenesis, cytokines, nitric oxide, intestine.

INTRODUCTION

Experimental African trypanosomiasis is characterized by a systemic inflammation with polyclonal lymphocyte activation, leading to multiple immune changes, including immunosuppression with hypergamma-globulinaemia (Vincendeau *et al*. 1999). The changes are initiated by substances from the parasites (see Tizard *et al*. 1978), especially the variant surface glycoprotein (VSG) and its glycosylphosphatidylinositol (GPI) anchor moieties (Tizard *et al*. 1978; Diffley, 1983; Magez *et al*. 1998). The contributions which the different parasite components contribute to the immune and pathogenic changes are not clear, but it has been demonstrated that separate fractions of the GPI anchor of *T.b. brucei* activate TNF-α induction, IL-1α induction and LPS sensitization in mouse macrophages (Magez *et al*. 1998).

Evidence also suggests that substances of non-parasite origin, which can activate immune changes, are produced in human and experimental trypanosomiasis. In particular, levels of endotoxins become markedly elevated in mice (Alafiatayo *et al*. 1993), rats (Nyakundi *et al*. 2002) and human patients (Pentreath *et al*. 1996). In patients the elevated levels are similar to those found in endotoxaemic conditions, with similar levels found in both plasma and cerebrospinal fluid (CSF) (Pentreath *et al*. 1996). The endotoxins appeared to be largely derived from Gram-negative bacteria (Pentreath *et al*. 1997). Studies on the rat model have shown that normal gut integrity is damaged during infection, with the levels of endotoxins in plasma correlating with the increased intestinal leakiness for much of the infection (Nyakundi & Pentreath, 1999; Nyakundi *et al*. 2002).

The significance of the endotoxins in the pathogenesis of trypanosomiasis is not understood, although by analogy with other diseases, it seems likely that they will contribute to the immune changes (Alafiatayo *et al*. 1993; Pentreath, 1994). Experimental animals infected with *T.b. brucei* develop a hyper-responsiveness to LPS (Magez *et al*. 1998), and considerably higher concentrations of VSG than LPS (i.e. μg VSG *cf* pg LPS) are required to evoke mitogenic responses, for example IL-1α production by mouse macrophages (Mathias, Perez & Diffley, 1990), which suggests that the increased LPS levels may be important in the immune responses.

We investigated the possible associations of the increased endotoxins in experimental rodent trypanosomiasis with the production of several cytokines and mediator substances already known to have important involvements in the disease. For this

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the studies were made on the same animals for which the intestinal leakage had been measured (see the accompanying article by Nyakundi *et al*. 2002), so that accurate comparisons between the blood cytokine levels, gut damage and endotoxins could be made. In addition, measurements were made of the production of cytokines and mediator substances by isolated intestinal tissues. These measurements were undertaken because firstly, the gut-associated lymphoid tissue (GALT) represents potentially one of the largest sources of cytokines and mediator substances in the body and secondly, because such substances can modulate the gut permeability. The following substances were measured: nitric oxide (NO) which kills trypanosomes and is involved in the immunosuppression (Vincendau *et al*. 1992; Beschin *et al*. 1998); TNF-α, which can also both control the levels of parasitaemia and cause immunopathology with immunosuppression (Magez *et al*. 1997, 1999); IL-1β, a pro-inflammatory monokine which is elevated in trypanosomiasis (see Rhind & Shek, 1999); IL-6, another predominantly pro-inflammatory cytokine which is involved in B cell proliferation and differentiation (see Vincendeau *et al*. 1999); and INF-γ, which is released by lymphocytes in response to, amongst other signals, substances released from the parasite, and which promotes parasite growth as well as being immunomodulatory in the disease (Bakhiet *et al*. 1996; see also Rhind & Shek, 1999; Vincendeau *et al*. 1999).

MATERIALS AND METHODS

Animals and trypanosome strains

Studies were made on male Wistar rats with *T.b. brucei* EATRO 1216 (kindly provided by Dr C. M. R. Turner of the University of Glasgow). This strain has relatively low virulence, causing a pathology which is threshold or low at day 7 and moderate/severe at days 21 and 33. The animals were infected by i.p. injections of 10^4–10^5 parasites in phosphate-buffered saline (PBS) and the parasitaemia monitored weekly. Animals were housed individually in metabolic cages with water and rat chow provided *ad libitum*, and maintained at 21 °C with a reverse 12 h light–dark cycle.

Some additional studies on NO production were made on adult BALB/c mice with GVR 35/c.1, which is also a strain with low virulence. These were made to give extra data on the time-course of NO production by the intestinal tissues in relation to the plasma levels, because the studies on rats were restricted to 2 sampling points (see below).

Sampling procedures

The experimental rats were part of a study analysing intestinal leakiness, circulating endotoxins and possible associations with cytokine and mediator substances (Nyakundi *et al*. 2002). The measurements using oral gavage of sugar probes were restricted to 2 per animal to avoid animal distress. Samples of blood and intestinal materials were correspondingly available at day 21 p.i. (i.e. sugar recovery measurements with gavage at days 10 and 21 p.i.) or day 33 (sugar recoveries at days 21 and 33).

Blood samples were taken via cardiac puncture under aseptic conditions immediately after sacrifice following the last permeability study. Blood samples were placed in endotoxin-free, heparinized tubes on ice, which were centrifuged at 180 ***g*** for 10 min, then stored at −20 °C until analysis. For some of the studies with mice, serum samples were obtained by allowing the blood to clot in sterile tubes before centrifuging at 400 ***g*** for 5 min before storage.

Intestinal biopsy samples were obtained immediately following the cardiac puncture. The intestine was dissected free from mesenteric tissue and the small intestine placed in a dish of saline. A 6 cm piece of jejunum was cut 3 cm from the end of the duodenum. The jejunum was selected because our previous studies (Nyakundi & Pentreath, 1999) had shown that this was markedly damaged during the infection. The piece of jejunum was flushed with sterile saline, then further divided into 2 cm lengths. These were diced into 1–2 mm square pieces which were incubated together in sterile dishes in medium consisting of RPMI 1640 (Gibco Ltd, UK) with 10% heat-inactivated FCS (Gibco Ltd, UK), 100 u/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 1 μg/ml Concanavalin A (ConA) (Sigma). The tissues were incubated for 24 h at 37 °C in humidified 95% air/5% CO_2 atmosphere, when the medium was removed, filtered and centrifuged (500 ***g*** for 10 min). These samples were stored at −80 °C until analysis.

Urine samples were collected from the animals in the metabolic cages at days 21 and 33 p.i. The urine was collected over a period of 8 h into collecting tubes containing 50 μl of thimerosal to prevent bacterial growth. The samples were frozen at −80 °C until analysis.

Endotoxin measurements

Endotoxins were measured by the quantitative chromogenic *Limulus* amoebocyte lysate (LAL) test (Coatest endotoxin kit, Endosafe Inc, USA). The sensitivity was 1–5 pg endotoxin/ml of plasma, measured against *Escherichia coli* serotype 0111.B4 (Sigma) as the standard.

Assay of NO products

NO combines quickly with oxygen to form nitrite and nitrate, which are relatively stable. The com-

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bined levels of these were measured after first reducing the nitrate to nitrite by fungal nitrate reductase. For this samples were diluted with nitrate-free distilled water (dilution 1:5 for plasma and serum and 1:20 for urine) and 100 μl of each were placed in a microtitre plate and incubated for 1 h at 37 °C with 1 unit *Aspergillus* nitrate reductase, 10 mg/ml reduced NADPH and 1 mg/ml flavine adenine dinucleotide (FAD). Excess NADPH was oxidized with L-lactic dehydrogenase type XI from rabbit muscle and 100 mM sodium pyruvate for 1 h at 37 °C. Then 100 μl of Griess reagent (0·5 % sulfanilamide and 0·05 % N-1-naphthyl-ethylenediamine hydrochloride in 2·5 % H_3PO_4) was added and after 5 min incubation at room temperature the absorbance was read at 570 nm. All the materials were purchased from Sigma. In some of the assays nitrite alone was measured, without conversion of the nitrate. Standard curves were prepared from sodium nitrate and sodium nitrite.

Cytokine assays

The levels of TNF-α, IL-1β, IL-6 and IFN-γ in the samples of plasma, sera, urine or culture supernatants were measured by solid-phase sandwich ELISA kits (Cytoscreen rat immunoassays; BioSource International Inc, USA). All measurements were made in triplicate in each bioassay with means calculated for each result; in practice S.E.s did not exceed 10 % with any assay.

Statistics

All results are expressed as the mean $\pm$ S.E.M. Student's *t*-test was used to determine the significances of the differences between the groups. Correlation–regression analysis was employed to determine the relationships between the levels of endotoxins, NO and cytokines with each other in the same and in the other fluid compartments analysed.

RESULTS

Blood endotoxin levels

Plasma endotoxin levels in rats were elevated approximately 3-fold at day 21, 4-fold at day 33 (Table 1).

NO production

The mean nitrate levels were raised approximately 10-fold in the plasma and 5-fold in the urine of infected rats at days 21 and 33 p.i. (Table 1). In mice the increases in blood nitrate were also large, with levels again raised 10-fold by day 7 p.i. (Table 2).

Nitrite production by the intestinal biopsy tissues (24 h cultures) of the mice were also significantly increased (Table 2), but in contrast to the blood the increases were not recorded before day 14 of the infections.

The correlations of the increases in NO products with other parameters measured in this study are described in the following sections.

Cytokine levels in blood and intestinal biopsy supernatants

The levels of TNF-α, IL-1β, IL-6 and IFN-γ were all significantly raised in the plasma of the infected rats at days 21 and 33 p.i. (Table 1). The highest increases were for TNF-α ($\times$7·0 at day 21, $\times$9·1 at day 33). Correlations between the cytokines and other parameters are described below.

Cytokine production by the cultured jejunum tissues of the rats were also significantly increased (Table 1). Again the highest increases were for TNF-α ($\times$5·7 at day 21, $\times$6·2 at day 33). Moreover the proportional increases for IL-1β, IL-6 and IFN-γ in the blood and intestinal tissues in the infected animals were very similar at each time-point. The levels of the cytokines in the intestinal supernatants were correlated with the blood levels (for TNF-α, $R = 0{\cdot}6$, $P < 0{\cdot}01$; for IL-1β, $R = 0{\cdot}69$, $P < 0{\cdot}01$; for IL-6, $R = 0{\cdot}68$, $P < 0{\cdot}01$; for IFN-γ, $R = 0{\cdot}55$, $P < 0{\cdot}01$). The cytokine studies in mice were limited to TNF-α and IL-6, for a smaller number of animals. However, the results showed that the increased production had commenced by day 7 p.i., with the increases at days 21 and 28 p.i. proportionally similar to those in the rats (Table 2).

Correlations between endotoxins, NO and cytokines

Because our measurements of the changes in gut permeability (see the accompanying article; Nyakundi *et al*. 2002), and levels of endotoxins, NO and cytokines were undertaken on the same animals, a search was made for possible correlations between them by correlation regression analysis. For this we included all the data points for each parameter and the control values at days 21 and 33 p.i.

Some of the relationships are summarized in Table 3. Of particular note were the exceptionally high correlations between the endotoxin levels and the cytokine levels in the plasma, and between the individual cytokines (examples in Figs 1 and 2). There were also strong correlations between the plasma endotoxins and NO, and the increased intestinal leakage. On the other hand the increases in plasma NO were not significantly correlated with the cytokines. There were also some significant correlations between the plasma endotoxins and the cytokine production by intestinal biopsies (for TNF-

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Table 1. Endotoxins, nitrate and cytokines in the blood and intestinal biopsies of rats infected with *Trypanosoma brucei brucei*

(Endotoxin and cytokine values are in pg/ml, nitrate values in μm. All values ±S.E.M., p.i., post-infection. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.)

	Control		Day 21 p.i.		Day 33 p.i.	
	n	Mean (±S.E.M.)	*n*	Mean (±S.E.M.)	*n*	Mean (±S.E.M.)
Blood						
Endotoxin	9	16·1 (1·62)	10	53·0 (10·60)**	10	70·5 (13·98)**
Nitrate	10	4·9 (1·96)	10	50·3 (3·58)***	8	51·8 (7·84)***
TNF-α	10	20·0 (6·32)	11	140·2 (47·40)*	9	181·4 (38·31)**
IL-1β	10	11·1 (3·80)	11	37·3 (10·48)*	9	52·7 (12·30)**
IL-6	10	33·5 (8·22)	11	144·5 (40·70)*	9	160·5 (49·5)*
IFN-γ	10	34·5 (9·49)	11	139·1 (40·0)*	9	113·3 (32·5)*
Urine						
Nitrate	10	11·6 (1·62)	10	50·8 (3·25)***	7	48·0 (9·81)*
Intestine						
TNF-α	10	4·7 (2·10)	8	27·0 (6·81)*	8	29·0 (5·30)**
IL-1β	10	6·2 (2·50)	8	22·3 (5·91)*	8	36·3 (8·52)**
IL-6	10	14·2 (3·61)	8	65·0 (14·1)*	8	51·9 (12·20)*
IFN-γ	10	9·8 (2·70)	8	43·6 (5·32)***	8	37·5 (8·81)*

Table 2. Nitrate, cytokines and endotoxins in the blood and intestinal biopsies of mice infected with *Trypanosoma brucei brucei*

(Endotoxin and cytokine values are in pg/ml, nitrate and nitrite values in μm. All values ±S.E.M., p.i., post-infection. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$, †Data from Alafiatayo *et al.* 1993.)

	Control		Day 7 p.i.		Day 14 p.i.		Day 21 p.i.		Day 28 p.i.	
	n	Mean (±S.E.M.)	*n*	Mean (±S.E.M.)	*n*	Mean (±S.E.M.)	*n*	Mean (±S.E.M.)	*n*	Mean (±S.E.M.)
Blood										
Nitrate	10	3·4 (0·51)	10	44·4 (7·80)***	10	23·7 (4·10)***	10	33·7 (3·30)***	10	23·8 (4·71)*
TNF-α	4	15·5 (1·76)	4	41·3 (12·5)**	4	50·1 (15·1)**	4	90·2 (18·3)***	4	161·6 (34·3)***
IL-6	4	15·0 (0·85)	4	78·7 (23·1)**	4	115·0 (21·6)***	4	247·5 (31·4)***	4	295·0 (37·0)***
Endotoxin†	18	14·25 (0·56)	18	36·4 (3·53)***	18	32·7 (2·96)***	23	36·4 (2·20)***	9	32·5 (3·99)***
Intestine										
Nitrite	10	5·5 (1·19)	10	5·1 (0·93)	7	31·2 (10·4)**	7	34·7 (10·2)**	7	21·9 (1·97)**

Table 3. Correlations between cytokines, NO and endotoxins in the plasma and intestinal permeability in rats infected with *Trypanosoma brucei brucei*

(The figures are the correlation–regression values (*r*) for all the data points for each parameter (including controls and days 21 and 33 p.i.), measured in the plasma of the same animals (source values of cytokines and endotoxins in pg/ml, NO in μM). The values in parentheses are the significances. Note the very high correlations between the endotoxin levels and several of the cytokines.)

	TNF-α	IL-1β	IL-6	IFN-γ	L/M Ratio	NO (plasma)
IL-1β	0·8 (< 0·01)					
IL-6	0·95 (< 0·01)	0·86 (< 0·01)				
IFN-γ	0·6 (< 0·01)	0·58 (< 0·01)	0·66 (< 0·01)			
L/M Ratio	0·54 (< 0·01)	0·50 (< 0·01)	0·58 (< 0·01)	0·32 (< 0·01)		
NO (plasma)	0·23 (N.S.)	0·32 (N.S.)	0·27 (N.S.)	0·29 (N.S.)	0·63 (< 0·01)	
Endotoxin	0·9 (< 0·01)	0·83 (< 0·01)	0·9 (< 0·01)	0·7 (< 0·01)	0·52 (< 0·01)	0·65 (< 0·01)

α, R = 0·56, $P < 0{\cdot}01$; for IL-1β, R = 0·58, $P < 0{\cdot}01$; for IL-6, R = 0·62, $P < 0{\cdot}01$; for IFN-γ, R = 0·62, $P < 0{\cdot}01$), between intestinal cytokine production and permeability (TNF-α, R = 0·42, $P < 0{\cdot}01$; IL-1β, R = 0·45, $P < 0{\cdot}01$; IL-6, R = 0·3, N.S.; IFN-γ, R = 0·55, $P < 0{\cdot}01$) and the production of cytokines

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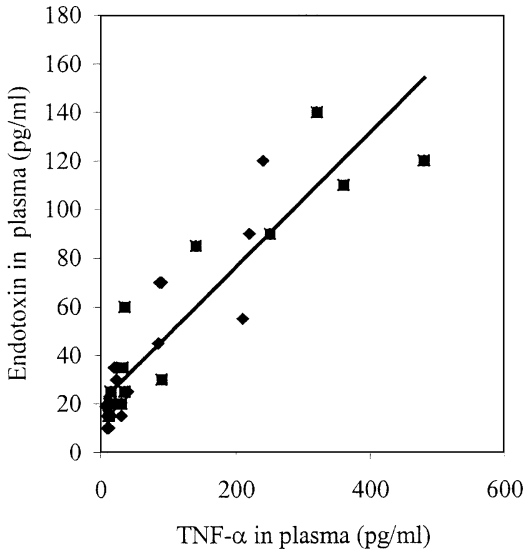

Fig. 1. Correlation between TNF-α and endotoxin in blood of rats infected with *Trypanosoma brucei brucei* ($r = 0{\cdot}9$, $P < 0{\cdot}01$). The data include controls and for days 21 (■) and 33 (◆) post-infection ($n = 29$).

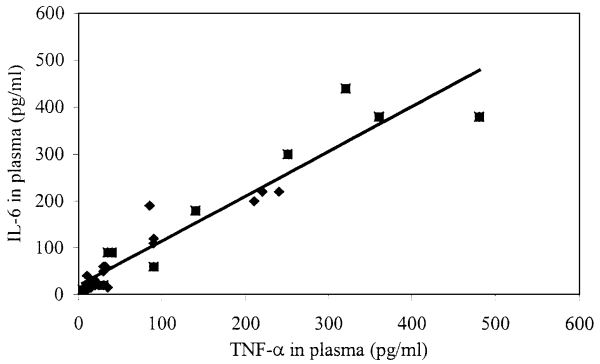

Fig. 2. Correlation between TNF-α and IL-6 in plasma of rats infected with *Trypanosoma brucei brucei*. The levels of cytokines were highly correlated ($r = 0{\cdot}95$, $P < 0{\cdot}01$). The data include controls and for days 21 (■) and 33 (◆) post-infection ($n = 30$).

and NO by the intestine (TNF-α, R = 0·64, $P<0{\cdot}01$; IL-1β, R = 0·48, $P<0{\cdot}01$; IL-6, R = 0·52, $P<0{\cdot}01$; IFN-γ, R = 0·56, $P<0{\cdot}01$).

A search was made to ascertain if any correlations conformed more accurately to log-transformed analysis. No increases in significance of the correlations were found by this procedure.

DISCUSSION

This study has provided good evidence for a strong association between the levels of circulating endotoxins and several cytokines and NO, previously shown to be perturbed in experimental trypanosomiasis. It also shows that the intestinal tissues may be an important site of the parallel production of these immunomodulators. Taken together with the findings presented in the accompanying article (Nyakundi *et al*. 2002), we conclude that increased intestinal leakiness during the infection is accompanied by elevated endotoxins which, in turn, are associated with the alterations in cytokines and NO.

The study raises a number of important issues concerning the nature and significances of the relationships. A comment is first required regarding the selection of the substances studied. Macrophage-derived NO is involved in the inflammatory responses mediated by endotoxins and cytokines, especially TNF-α. Both increases (Sternberg & McGuigan, 1992) and decreases (Buguet *et al*. 1996) have been reported in laboratory models. NO is trypanostatic *in vitro* (Vincendeau *et al*. 1992), but this effect may be diminished in the presence of blood as occurs *in vivo* (Mabbott, Sutherland & Sternberg, 1994). In addition to its likely protective role against the parasite, it has been clearly demonstrated that NO can partially contribute to the suppression of T-cell responses during the disease (Sternberg & McGuigan, 1992; Schleifer & Mansfield, 1993; Beschin *et al*. 1998; Millar *et al*. 1999). The involvement of TNF-α in trypanosomiasis has been studied. TNF-α is trypanolytic and kills the parasites *in vitro* (see Magez *et al*. 1999). High levels correlate with disease severity in man (Okomo-Assoumou *et al*. 1995). IL-1 increases in trypanosomiasis may be induced by components of the parasite surface coat (Sileghem *et al*. 1989; Mathias *et al*. 1990). IFN-γ production by $CD8^+$ T cells is polyclonally triggered by trypanosome substances, notably a released substance termed trypanosome lymphocyte triggering factor (TLTF; see Bakhiet *et al*. 1993). Unlike many other protozoal infections, IFN-γ can act as a virulence factor by promoting growth of *T. brucei* (Bakhiet *et al*. 1990, 1996). Less is known about the possible involvement in trypanosomiasis of another largely pro-inflammatory cytokine, IL-6 (see Vincendeau *et al*. 1999), although it has been demonstrated to be significantly up-regulated in the brains of mice infected with *T.b. brucei* (Hunter *et al*. 1991). The possible associations of the cytokines and mediator substances amongst themselves, with other immunoregulators, and their cellular sites of production in trypanosomiasis have been examined (see reviews by Rhind & Shek, 1999 and Vincendeau *et al*. 1999). Studies of the relationships of endotoxins with other cytokine/mediator substances than those examined in the present work may be worthwhile.

A key issue regards the significance, or otherwise, of the correlations discovered in the present study in the disease pathogenesis. The extremely high correlations between the circulating endotoxins and the cytokines and NO provide direct evidence that the former are a significant component of the immune regulatory changes. However, the data do not provide information on the causative relationships; on the one hand the intestinal damage with increased

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endotoxins could be a consequence of the elevated cytokine/mediator substances occurring systemically because of the parasite and acting via the intestinal blood supply. On the other hand the intestinal damage may be a primary event which initiates, in part, immune alterations elsewhere in the body. The present and previous studies (Alafiatayo *et al*. 1993) were restricted to days 7, 21 and 33 days p.i. in rats and mice for the different parameters, with similar changes generally recorded on each occasion. There was one exception; NO production by the intestinal biopsies of mice was not increased at 7 days p.i., whilst NO products and endotoxin levels in the blood of the same animals were significantly increased. It is possible that further experiments employing measurements of the different parameters separated by several hours during the early stages of infection may resolve this. Such measurements should include close attention to measurements of parasitaemia, since this might shed light on the relative contributions of endotoxins and parasite moieties on the induction of cytokine/mediator substances. In relation to this it has been shown that there is a prompt production of IFN-γ, NO and TNF-α by the first increases in circulating parasites, although the increases in the cytokines and NO may not subsequently be maintained nor correlate with subsequent waves of parasitaemia (Bancroft *et al*. 1983; Mabbot *et al*. 1998; Magez *et al*. 1998; Rhind & Shek, 1999). However, such multiple analyses could be technically very difficult on laboratory rats and mice, and interpretation could be complicated by the unknown significances of parasites inside the tissue compartments which may not correlate with parasitaemia.

It is also not yet clear which substances or signals may induce the increased intestinal leakiness, allowing an altered movement of substances into the blood. NO is one of a number of substances involved in the regulation of gastric integrity (Whittle, Lopez-Belmonte & Moncada, 1990), as well as its alterations in pathological situations involving systemic endotoxin challenge (Hutcheson, Whittle & Boughton-Smith, 1990) and isolated intestinal tissues (Boughton-Smith *et al*. 1993*a*). There are substantial increases in inducible NO synthase (iNOS) in the colonic mucosa and NO products in the blood of patients with inflammatory intestinal disorders such as ulcerative colitis or Crohn's disease (Boughton-Smith *et al*. 1993*b*). In addition a number of cytokines, including IL-1, IL-6 and IFN-γ may activate iNOS and contribute to the altered intestinal integrity in inflammatory intestinal disorders (Isaacs, Sartor & Haskill, 1992). The extensive lymphoid tissue in the gut (GALT) can be a major producer of a range of cytokine/mediator substances, with the macrophage a likely key producer in inflammatory disorders (Reimund *et al*. 1996). In experimental trypanosomiasis the GALT becomes enlarged with inflammatory infiltrations (Nyakundi & Pentreath, 1999). There are furthermore a potentially very large number of substances liberated in trypanosomiasis which can modify endothelial integrity throughout the animal (see Pentreath, 1999; Tizard *et al*. 1978). Once again there is great difficulty in unraveling the sequences of cause and effect in the activities and associations of the substances in the correlations discovered in the present study.

The extents to which the elevated endotoxins may initiate the alterations in cytokines are not yet clear. A range of trypanosome moieties and released substances are also known to be strong inducers of cytokine production, especially the surface (VSG) coat (Mathias *et al*. 1990). Different components of the VSG may initiate different immune responses (Magez *et al*. 1998). However, endotoxins are among the most potent inducers of cytokine production, for many cell types being effective in pg/ml concentrations, which can be several orders of magnitude less than the concentrations of *T.b. brucei* material (μg/ml or mg/ml) required to cause similar activations. Endothelial damage to the microvasculature in trypanosomiasis may be mediated partly by parasite phospholipases and several biologically active peptides (kinins) generated in the infected animals (see Tizard *et al*. 1978). Additionally, the procedure we employed for measuring endotoxins evaluates several bacterial factors including LPS (which is the most potent mitogenic component) and these together with other substances (e.g. viral and yeast products) which are not measured but which reach the circulation as a result of the altered intestinal integrity may also stimulate cytokine synthesis (see Henderson & Wilson, 1996).

An obvious experimental approach might appear to be the study of the cytokine/mediator production in LPS-sensitive or LPS-resistant animals (e.g. C_3H/HeN, C_3H/HeJ mice), compared to the wild-type animals used in the present study. Such animals have already been used in a number of studies on trypanosomiasis, for example the LPS-sensitization which accompanies the disease (Magez *et al*. 1998), but there are no reports of significant differences in mortality. Complicating factors here are the multiple time-dependent effects and pathways by which endotoxin can act; for example mice which are CD14-deficient (i.e. lack the principle LPS receptor expressed on monocyte/macrophages) are resistant to endotoxin shock but may still generate inflammatory cytokines via other receptors after prolonged LPS exposure (Haziot *et al*. 1996).

In conclusion, the elevations in circulating endotoxins which are a feature of both human and experimental trypanosomiasis, are accompanied in the laboratory animal model with correlated increases in NO and inflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ), with an involvement of

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compromised intestinal tissue. The contributions of the endotoxins and the significance of these correlations in the disease pathology require elucidating.

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