The relationships between intestinal damage and circulating endotoxins in experimental *Trypanosoma brucei brucei* infections

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SUMMARY

The involvement of intestinal damage in experimental African trypanosomiasis was investigated in rats infected with *Trypanosoma brucei brucei* by measuring the urinary excretion of the previously administered non-metabolizable sugar probes, D-mannitol and lactulose, and the flux of FITC-dextran across isolated, everted gut segments. There was increased urinary recovery and flux of the sugar probes across the intestine which were significant ($P < 0{\cdot}05$) and maximum at day 21 of the infection, but subsequently reduced, in the terminal stages of infection (day 33 p.i.). In the case of the everted sac studies the reductions were to less than 25 % control values ($P < 0{\cdot}001$). Levels of circulating endotoxin were increased approximately 3-fold at day 21 p.i., 4-fold at day 33 p.i., compared to controls. At day 21 there was a significant correlation ($r = 0{\cdot}63$, $P < 0{\cdot}01$) between the log endotoxin levels and the increased sugar excretion expressed as the lactulose/mannitol ratios. Histological studies showed damage to the villi, wall thinning and marked cellular infiltrations, which were very prominent in the proximal jejunum and duodenum. These results demonstrate that during trypanosome infections in rats, increased intestinal leakage and increased circulating endotoxins are significant pathological features.

Key words: experimental African trypanosomiasis, *Trypanosoma brucei brucei*, endotoxin levels, cytokines.

INTRODUCTION

In both experimental and human African trypanosomiasis there are large increases in circulating endotoxins (Alafiatayo *et al*. 1993; Pentreath *et al*. 1996, 1997). The endotoxins are largely derived from Gram-negative bacteria, which raises the possibility that they could originate from the intestine (Pentreath *et al*. 1997). In mice infected with *T. b. brucei* there are marked structural changes in the intestinal wall, especially the small intestine where there is disruption of the villi, cellular infiltration of the mucosal and submucosal layers and oedema of the lamina propria and lymphatics (Nyakundi & Pentreath, 1999). Morrison *et al*. (1981) noted pathological changes in a wide range of tissues, including stomach and intestine, in dogs infected with *T. b. brucei*.

Because of the potential importance of the elevations in endotoxins and the morphological changes in the gut in contributing to the pathology (Pentreath, 1994; Pentreath *et al*. 1996), we undertook further studies in the rodent model. The purpose was to measure the permeability of the intestine during infection with *T. b. brucei*, and to assess whether any alterations in this correlated with increases in circulating endotoxins levels. Intestinal permeability was evaluated by the urinary excretion of the non-metabolizable sugar probes, D-mannitol and lactulose (Travis & Menzies, 1992), and the everted gut sac techniques (Carter *et al*. 1990). In the accompanying article (Nyakundi, Crawley & Pentreath, 2002) we extend the findings to evaluate further possible correlations between permeability changes, increases in circulating endotoxins and alterations in a range of cytokine/mediator substances in the infected animals.

MATERIALS AND METHODS

Animals and trypanosome strains

The intestinal permeability experiments were carried out on male Wistar rats and 2 different strains of *T. b. brucei*; GVR35/c.1 and EATRO 1216 (kindly provided by Dr F. Jennings and Dr C. M. R. Turner of the University of Glasgow). Both strains have relatively low virulence with the animals surviving more than 30 days. The rats were infected by intraperitoneal infection of 10^4–10^5 parasites in phosphate-buffered saline (PBS) and the parasitaemia monitored weekly. After infection, rats were housed individually in wire-framed metabolic cages

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and received water and rat chow *ad libitum*. The animals were maintained at 21 °C with a reverse 12 h light–dark cycle and were weighed twice a week.

Food and water measurements

Normal intestinal barrier function is dependent on a balanced diet (Baumgartner & Cerda, 1993). An absence of normal intestinal stimulation by lack of food intake in starvation or total parental nutrition causes marked morphological and biochemical changes (Carey, 1992; Illig *et al*. 1992), decreased barrier function and increased translocation of bacteria and their products (Alverdy, Aoys & Moss, 1988). Therefore food and water consumption by the experimental animals was carefully monitored. Daily food consumption was calculated from the differences between weights given and remaining (including food spilled). Water consumption, faeces and urine production were also monitored daily. Any animal with any indication of altered faeces, especially diarrhoea was excluded from the study.

Intestinal permeability by oral gavage of sugars

The non-invasive study of intestinal permeability, using the dual sugar test, is extensively used for assessing gut integrity in a range of animals including rats (Turner *et al*. 1988) and is commonly used for patients in a clinical setting (e.g. Travis & Menzies, 1992). When the non-metabolizable sugars are taken orally they are poorly absorbed, but when the mucosal barrier is disrupted the markers cross the gut, enter the circulation and are excreted in the urine. Different sized probes are absorbed by different routes. The frequently used larger molecule lactulose (MW 342) is absorbed predominantly via the paracellular pathway, whereas the smaller D-mannitol (MW 142) is absorbed predominantly transcellularly. In practice, gut permeability is additionally expressed as the permeation ratio of large: small molecules, since this provides an indication of the total changes taking place and it reduces variations due to non-mucosal factors, including gastric emptying, intestinal transit, intestinal dilution and renal function, which may affect the results (see for example, Turner *et al*. 1988). For both practical and ethical reasons the number of permeability tests with gavage on each animal was limited to 2. Tests on different animal groups were undertaken before infection, and at days 10, 21 or 33 post-infection. These days were selected in relation to the infection-associated pathology caused by the low virulence parasite strains, which is threshold or low at day 10, and moderate/severe at days 21 and 33. Before each test, animals were fasted overnight but had free access to water. Twelve h before the administration of sugars the urine was collected to test for the possible presence of native sugars. The rats were gavaged with 2·5 ml of test solution containing 50 mg D-mannitol and 66 mg lactulose (Sigma Chemical Co Ltd, Poole, UK). The test solution was, therefore, isotonic (245 mosm/l), although initial experiments with both hypertonic and hypotonic solutions had shown no effects of osmolarity on the excretion of sugars. At the start of the experiments 60 μl of 1 % thimerosal solution was put into each urine collection container to prevent bacterial consumption of the sugars. The urine was collected over 8 h. The total volume of urine was recorded and a sample stored at −20 °C for subsequent sugar analysis.

Lactulose and mannitol in the urine samples were quantified by separation by high performance liquid chromatography (HPLC) and pulsed amperometric detection, with use of a Dionex Carbopac PA1 and guard column.

Depending on the collection volume rat urine samples and controls were diluted between 1:4 and 1:16 with deionized water to a volume of 1 ml. Then 15 μl of internal standard (Cellobiose) were added and the mixture was desalted with ion-exchange resin taking up to 30–50 % of the total volume. The standards (known mannitol and lactulose concentrations) were also prepared in a similar way except that these were not desalted. Finally 4·9 ml of deionized water were put in a sampling tube and 120 μl of standard or desalted urine sample were added. The cap was fitted and mixed by inversion and tapped to remove air bubbles. This was then placed on HPLC. Samples of 10 μl were injected onto the column. The samples were eluted with NaOH:zinc acetate eluent at a rate of 1·5 ml/min at room temperature. The eluent was prepared as follows: 800 ml of deionized water were degassed with helium for 20 min and to this were added 325 μl of zinc acetate and 4·9 ml of 46/48 % NaOH. All samples were analysed with a pulsed electrochemical detector and peak heights measured with internal standardization.

Calculation of sugar recoveries and statistical analysis

Concentrations of sugars were measured against a standard curve and recoveries calculated as a percentage of the amounts of water ingested. Permeabilities of the animal intestines were expressed as the ratios of the recoveries of lactulose and mannitol. The data were expressed as mean ± S.E.M. Non-parametric tests (Kruskal Wallis) were used. To evaluate the treatment groups that differed in size from the others the Mann-Whitney test or Dunn's multiple comparison procedure were implemented.

Intestinal permeability by everted sac technique

The analysis of the movement of substances across the intestine has been aided by the use of isolated intestinal segments which have been turned 'inside-

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out' (everted sac). This *in vitro* method allows analysis of different levels of the gut and is not limited by a number of physical factors including gut transit and emptying, blood flow and renal function (Carter *et al*. 1990), although as with all *in vitro* methodologies other limitations apply (e.g. damage during the isolation procedure).

Animals were terminally anaesthetized after the collection of urine for sugar measurements, at different stages (i.e. days 21 and 33 p.i.) of the infections. Eight cm lengths of small intestine, approximately 3 cm from the end of the duodenum were rinsed in 20 ml of ice-cold saline, and the segments carefully everted with a glass rod. The everted segment was ligatured at one end. The sacs were filled via glass cannulae from the open end with 1 ml of 0·05 m phosphate buffer in 0·9 % saline solution, pH 6·5, and the end also tied off. The segments were placed in dishes containing 25 ml of phosphate saline containing 0·05 mg/ml fluorescein isothiocyanate (FITC) – labelled dextran (average MW 4400), and these were incubated for 1 h at 37 °C in a metabolic shaking bath, with 95 % O_2/5 % CO_2 bubbled into the solution. After 1 h the segments were removed, rinsed with phosphate buffer and gently blotted. The segments were emptied and the amounts of FITC-dextran in their contents diluted in steps in 0·05 M phosphate buffer in 0·9 % saline (pH 6·5). Samples from the external bathing medium (i.e. serosal side of the intestine) were also diluted in steps, and all FITC-dextran concentrations measured by spectrofluorimeter (excitation wavelength 475 nm, emission wavelength 515 nm). Comparisons were made against a standard curve to assess the permeation of the FITC-dextran through the intestine.

Endotoxin measurements

At the time of the last permeability study by sugar excretion and everted gut segment analysis on days 21 or 33 p.i., blood was collected for analysis of endotoxin content. Great care was made to ensure aseptic collection. Following terminal anaesthesia the skin overlying the thorax was shaved, at the same time being sterilized with alcohol swabs. The heart was exposed, again with alcohol swabbing, and blood collected via cardiac puncture using a 5 ml syringe with 23 gauge needle. The blood was placed immediately in endotoxin-free heparinized tubes on ice and centrifuged at 180 ***g*** for 10 min at 4 °C. The plasma was stored at −20 °C with analysis.

Endotoxins were quantified by the *Limulus* amoebocyte lysate (LAL) test. The plasma samples were heated at 75 °C for 10 min to inactivate possible heat-labile, interfering substances or plasma inhibitors. Endotoxin activity was measured using a chromogenic modification of the LAL test (Coatest endotoxin kit, Endosafe Inc, USA). The sensitivity was 1–5 pg endotoxin/ml of sample fluid, measured by *Escherichia coli* serotype 0111.B4 (Sigma) as standard. The endotoxin levels in the plasma of control and infected animals were compared by Student's *t*-test.

Histology

Our recent studies on the morphological changes in the intestine caused by *T. b. brucei* infection were undertaken on the mouse (Nyakundi & Pentreath, 1999), but there are no comparable data on the rat model. We therefore preserved pieces of the small intestine at the end of the experiments described above by fixing in phosphate-buffered 10 % formalin for 24 h. The pieces were then cut into 6–8 mm lengths and processed histologically according to the standard procedures (Nyakundi & Pentreath, 1999).

RESULTS

Food and water intake and animal weight

Both the control and the infected animals consumed a smaller amount of food (less than 10 %) during their first week in the metabolic cages. However, there was no significant difference between the control and experimental animals at this stage and the alteration in both groups may result from the change in normal environment with novel food compartments. There was no further significant difference in food intake between the groups until after day 21, when the infected animals had reduced intake (10–20 %). Both groups consumed the same amount of water throughout the experiments. The weight added by both groups was not significantly different for the first 3 weeks of the infection, but subsequently some infected rats started to lose weight, especially in the terminal stages.

Intestinal permeability assessed by sugar recoveries

The results of the 2 low-virulence strains of *T. b. brucei* used were very similar. For both strains the pathologies were not significantly developed until 2 weeks p.i. Very few infected animals survived beyond the sixth week of infections and some deaths occurred by day 33. In order to compare the 2 strains animals were studied in groups of 10, with *T. b. brucei* strain GVR35/C.1 studied at days 10 and 21 p.i., and the strain EATRO 1216 at days 21 and 33 p.i.

The results are summarized in Table 1. There were increases in the lactulose and mannitol recoveries and L/M excretion ratios at all times during the infections, but these were significant only at day 21 p.i. These increases in intestinal permeability were maximum at this time, and were reduced at day 33.

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Table 1. Lactulose and mannitol recoveries and lactulose/mannitol (LM) ratios in rats infected with *Trypanosoma brucei brucei*

(Lactulose and mannitol recoveries expressed as percentages, all values ± S.E.M.; * denotes $P < 0{\cdot}05$ compared to control by Student's *t*-test; p.i., days post-infection.)

	Control		Day 10 p.i.		Day 21 p.i.	
Strain *T. b. brucei*	GVR 35/c.1	EATRO 1216	GVR 35/c.1	EATRO 1216	GVR 35/c.1	EATRO 1216
N	9	9	9	–	8	10
Mean lactulose recovery (%) (S.E.M.)	1·8 (0·23)	1·6 (0·31)	3·4 (1·17)	–	4·43 (1·13)*	4·5 (0·95)*
Mean mannitol recovery (%) (S.E.M.)	4·4 (0·64)	4·6 (0·59)	5·6 (0·78)	–	6·36 (0·88)*	9·1 (1·43)*
L/M ratio	0·4 (0·01)	0·3 (0·03)	0·5 (0·09)	–	0·65 (0·08)*	0·48 (0·03)*

Table 2. FITC-dextran permeation across everted segments of small intestine of rats infected with *Trypanosoma brucei brucei*

(Values are for FITC-dextran movement across 8 cm lengths of small intestine per h; * denotes $P < 0{\cdot}001$ compared to control by Student's *t*-test; p.i., days post-infection.)

	Control	Day 21 p.i.	Day 33 p.i.
N	10	6	7
Mean FITC-dextran (S.E.M.)	9·7 (1·30)	13·8 (1·86)	2·2 (0·21)*

Table 3. Endotoxin levels in the plasma of rats infected with *Trypanosoma brucei brucei*

(Values are the mean ± S.E.M. in pg/ml; * denotes $P < 0{\cdot}01$ compared to control by Student's *t*-test; p.i., days post-infection.)

	Control	Day 21 p.i.	Day 33 p.i.
N	9	10	10
Mean endotoxin (S.E.M)	16·1 (1·62)	53·0 (10·60)*	70·5 (13·98)*

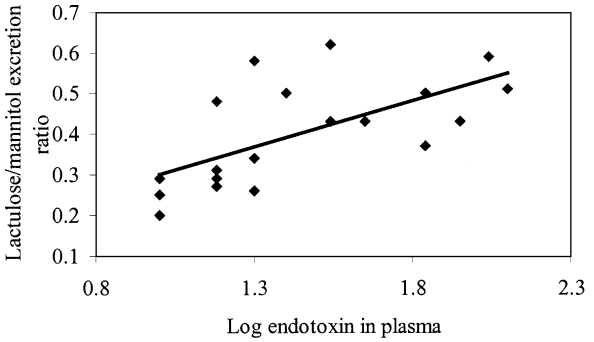

Fig. 1. Correlation between endotoxins in the plasma and intestinal permeability (expressed as the lactulose/mannitol excretion ratio) in rats infected with *Trypanosoma brucei brucei* at 21 days post-infection. Endotoxin and permeability ($r = 0{\cdot}55$, $P < 0{\cdot}01$) and log endotoxin and permeability ($r = 0{\cdot}63$, $P < 0{\cdot}01$).

Intestinal permeability assessed by everted sac technique

The permeation of FITC-dextran was assessed in rats with *T. b. brucei* strain EATRO 1216 at days 21 and 33 p.i. The movement of the tracer across the intestine was increased at day 21 p.i., but this was not statistically significant. However, at day 33 p.i. the FITC-dextran permeation was markedly reduced ($P < 0{\cdot}001$) (Table 2). Moreover, this reduction was so great that the intestinal permeability became considerably less than the control values.

Endotoxin levels in the plasma and correlation with intestinal permeability

The levels of endotoxin in the plasma became progressively raised during the infection (Table 3). The mean levels were approximately 3-fold control values at day 21, 4-fold at day 33.

The plasma endotoxin levels and intestinal permeability (expressed as the L/M excretion ratios) in the control and infected animals were studied by correlation-regression analysis. At day 21 p.i. there was a moderate correlation ($r = 0{\cdot}55$, $P < 0{\cdot}01$), especially when the plasma endotoxin was expressed in log units ($r = 0{\cdot}63$, $P < 0{\cdot}01$; Fig. 1). At day 33 p.i. there was no significant correlation ($r = 0{\cdot}4$).

Histological findings

There were some marked structural changes in the intestinal wall at the different levels of the small and large intestine. The alterations were very similar to those recently reported for mice infected with the same strains of *T. b. brucei* (Nyakundi & Pentreath, 1999). The changes were very prominent in the proximal jejunum and the duodenum, with wall thinning, reductions in villous height/width ratios, cellular infiltration of the mucosal and submucosal layers with expansion of lamina propria and lymphatics. At the terminal stages (i.e. day 33 p.i.) the villi tips were frequently swollen and oedematous.

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DISCUSSION

This study demonstrates that *T. b. brucei* infections in rats are associated with increased intestinal permeability when measured by the methods of L/M excretion and everted gut segments. In the same rats there are associated increases in circulating levels of endotoxins. The findings raise important issues concerning the disease pathogenesis and the associated alterations in immune signalling by cytokine/mediator substances, which are analysed further in the accompanying article (Nyakundi *et al*. 2002).

The L/M test has been widely used to investigate intestinal permeability in a range of experimental and disease conditions in laboratory animals and man. The simultaneous administration of two probes and the expression of urinary recoveries as a ratio reduces the influence of factors such as gastric emptying, intestinal dilution, intestinal transit, renal function and incomplete urinary recovery. L/M ratios are also influenced by other factors such as starvation, mode of nutrition and diarrhoea from different causes. In the present study these factors, where possible, were minimized. Food intake by the infected rats was not significantly different from the controls until the late stages of infection, by which time significant alterations in L/M ratio had already taken place. Renal function was not measured, but there were no changes in the volume of urine excreted by the infected animals and this is therefore unlikely to be an interfering factor. The control sugar recoveries were similar to those reported in other studies on rats (for example, Pernet *et al*. 1998), and there are some differences from man (for example, Noone *et al*. 1986; Andre *et al*. 1987).

The increases in both lactulose and mannitol recoveries in the present study indicate that both transcellular and paracellular permeation routes are affected. It is widely thought that mannitol (MW 142) recovery reflects the transcellular route and relates to villous damage, whereas lactulose (MW 342) reflects changes in the tight junctions and membrane integrity with increased access of the luminal contents to the crypts (Travis & Menzies, 1992). The alterations in the sugar recoveries and their ratios have been studied in situations which include burn injury (Pernet *et al*. 1998), coeliac disease (Catassi *et al*. 1997), intestinal damage (Cobden, Rothwell & Axon, 1981), food allergy (Andre *et al*. 1987), trauma (Pape *et al*. 1994), Crohn's disease (Pearson *et al*. 1982) and experimental infestation in rats by *Nippostrongylus brasiliensis* (Cobden, Rothwell & Axon, 1979). Each of these induce their own patterns of altered permeability, but the changes in sugar excretions in the present study compare most closely to those caused by systemic administration of endotoxin in healthy humans (O'Dwyer *et al*. 1988).

The results of sugar recoveries were complemented by the everted sac studies. The size of the dextran we employed (MW 70000) is larger than that used in some other reports (for example, Horie, Nakamaru & Masabuchi, 1998), but will indicate severe mucosal disruption, especially of the paracellular routes. The everted sac experiments are prone to methodological errors, especially damage caused by the everting procedure, but we took great care to minimize these.

Although both methodologies and histological studies provide consistent strong evidence for increased intestinal permeations and damage caused during the infection, there were apparent inconsistencies in the later, pre-terminal stage, when the sugar recovery measurements showed return to control values and, more surprisingly, the everted sac measurements marked reductions in permeation. The reasons for the reversals are not clear, although in the case of the everted sac studies it is possible that severe intestinal disruption may be associated with some back diffusion of the dextran probe. However, it seems possible that in the terminal disease stages the severely damaged intestine may convert to a refractory state which protects against the wholesale transfer of substances, but with some levels of circulating endotoxins (which have not been cleared by the liver) still high.

The increases in circulating endotoxins during the infections extended earlier studies on the mouse model (Alafiatayo *et al*. 1993). The control values in both rat and mouse are similar, but slightly higher than those reported for man (McCartney *et al*. 1987; Pentreath *et al*. 1996). The possible involvement of endotoxins in African trypanosomiasis has been previously discussed (Alafiatayo *et al*. 1993; Pentreath, 1994; Pentreath *et al*. 1996, 1997). The significant correlations between the plasma endotoxins and L/M ratios at day 21 found in the present study confirms our previous suggestions of a likely gut origin (Pentreath *et al*. 1996). However, the weaker relationship between endotoxin levels and permeability at the later stages of infection (i.e. after day 33 p.i.) strongly indicate that other factors, perhaps associated with protection against transintestinal fluxes, became important. Increased circulating endotoxin levels, often associated with endotoxaemia in the absence of specific septic focus, and increases in intestinal permeability with disruption of the intestinal mucosa occurs in a range of conditions including trauma (Deitch & Bridges, 1987; Border *et al*. 1987) and inflammatory conditions such as inflammatory bowel disease and Crohn's disease (e.g. Aoki, 1978; Bjarnson *et al*. 1983).

Endotoxins that cross the gut mucosa are cleared by several mechanisms including leucocyte receptor-mediator events, bactericidal/permeability increasing protein (BPI) released from neutrophils, cir-

culating antibodies and the Kupffer cells in the liver following their transport from the gut mucosa via the portal vein (see Corriveau & Danner, 1993; Galanos & Freudenberg, 1993; Pentreath *et al*. 1997). It is not clear to what extent these symptoms may be compromised in trypanosomiasis, although it is well known that the infection is associated with chronic hepatomegaly as the Kupffer cells clear trypanosome materials.

The mechanisms by which *T. b. brucei* bring about the alterations in intestinal permeability are also not yet clear. Possible causes include a number of cytokines and/or mediator substances, acting either from the circulation or produced locally in the inflammatory sites of cellular infiltration in the intestinal tissues. Candidates include TNF-α and NO, known to be elevated in trypanosomiasis (see Rhind & Shek, 1999). In particular NO, if produced in excess can potently modify epithelial permeability (Salzmann, 1995). The production of these substances by the isolated intestinal tissues of rats infected with *T. b. brucei* is discussed in the accompanying article (Nyakundi *et al*. 2002).

Thus elevated circulating endotoxins associated with altered gut integrity appears to be a significant component of the pathology of *T. b. brucei* infections in the rodent model. A number of other parasite substances, including components of the variant specific glycoprotein surface coat, have already been established as contributing to the immune changes in the disease (e.g. Magez *et al*. 1998; see also Tizard *et al*. 1978 for a review of the early studies), although endotoxin may be effective at much lower concentrations (Mathias, Perez & Diffley, 1990). The contributions of the different substances in the disease pathogenesis require clarification.

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