Endotoxin antibodies in African sleeping sickness

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SUMMARY

Antibodies to the core region of endotoxin (endotoxin core antibodies, EndoCAb), which cross-react with endotoxin from a range of Gram-negative bacteria, are maintained in relative homeostasis in health, but undergo marked changes in a number of different diseases associated directly or indirectly with endotoxaemic or septicaemic states. The levels of EndoCAb IgG in the blood and cerebrospinal fluid (CSF) of 35 late-stage sleeping sickness patients and 9 control individuals were measured by ELISA. EndoCAb levels were significantly elevated in the patient blood (mean EndoCAb value 290 MU/ml *cf*. control 182 MU/ml, $P < 0{\cdot}001$), and CSF (mean EndoCAb value 254 MU/ml *cf*. control 150 MU/ml, $P < 0{\cdot}001$). EndoCAb IgG levels correlated with endotoxin levels in patient blood ($r = 0{\cdot}78$, $P < 0{\cdot}001$), but not in the CSF and were not reduced 6 weeks following chemotherapy, unlike the endotoxin levels. It is concluded that late-stage sleeping sickness is associated with chronic exposure to endotoxins from Gram-negative bacteria.

Key words: African sleeping sickness, endotoxin core antibodies, pathogenesis, cerebrospinal fluid.

INTRODUCTION

Endotoxin levels become markedly elevated in the blood and cerebrospinal fluid (CSF) of African sleeping sickness patients, are reduced 6 weeks following chemotherapy and both before and after treatment there is close correlation between the levels in the two fluid compartments (Pentreath *et al*. 1996). Similar large increases in serum endotoxin have been recorded for *T. b. brucei* infections with the mouse model (Alafiatayo *et al*. 1993). Because comparable increases in endotoxin in other septicaemic or disease states can trigger widespread pathological changes, it has been suggested that the raised endotoxin may contribute to the pathology in sleeping sickness (Greenwood, 1974; Pentreath, 1994; Pentreath *et al*. 1996).

Circulating endotoxin is detoxified in several organs, principally the liver, but the effects may be strongly modulated by other clearance mechanisms (Corriveau & Danner, 1993; Galanos & Freudenberg, 1993). High density lipoprotein (HDL) and to a lesser extent low density lipoprotein (LDL) bind and inhibit LPS, although the effect is attenuated if outer membrane proteins (Omp) are attached to the LPS. Another binding protein, LPS binding protein (LBP), complexes with lipid A and facilitates binding of endotoxin and Gram-negative bacteria to macrophages, monocytes and neutrophils via the CD14 receptor, thus triggering the defence cascade more strongly than LPS alone. CD14 receptors on the cell surface of myeloid cells bind the LBP–LPS complex and this initiates production of pro-inflammatory mediators. Soluble forms of CD14 are present in normal serum. Neutrophils also contain and, inflammation may release, bactericidal/permeability increasing protein (BPI). This protein is antibacterial to Gram-negative bacteria and binds to and inhibits LPS. An additional important mechanism of inactivation is by antibodies to the core oligosaccharide of the LPS (endotoxin core antibodies, EndoCAb; see Barclay, 1995), many of which are cross-reactive and probably offer general protection against endotoxin. EndoCAb of the different antibody classes is common and occurs in substantial quantities in the sera of most healthy adults. It is likely that its production is maintained through the constant exposure to the normal low levels of circulating endotoxin. EndoCAb levels vary significantly in a number of clinical and endotoxaemic conditions. The levels have a high degree of clinical relevance, both as marker of endotoxin exposure and a prognostic indicator (Barclay, 1995; Poxton, 1995).

The purpose of the present work was to investigate the levels of EndoCAb in the blood and CSF of a group of sleeping sickness patients diagnosed as having CNS involvement at the Project de Recherches Cliniques sur la Trypanosomiase

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(PRCT), Daloa, Côte d'Ivoire. The levels were compared with those in the same patients following chemotherapy, and with controls. Possible correlations between EndoCAb, endotoxin and other markers in the blood and CSF were studied in order to assess the effects of endotoxin exposure in the chronic disease state.

MATERIALS AND METHODS

Patients

Studies were made on a group of 35 late-stage sleeping sickness and a group of 9 controls without evidence of trypanosomiasis. Diagnostic tests were undertaken at the PRCT, Daloa. Most of the late-stage patients had been part of our previous study on endotoxin levels (Pentreath *et al*. 1996). The tests were evaluation of serum by the card agglutination test for trypanosomes, the identification of trypanosomes in peripheral blood or lymph gland aspirate by the microhaematocrit centrifuge technique or the mini-anion-exchange centrifugation technique, and by a range of clinical manifestations, as have been previously described in detail (Pentreath *et al*. 1996). CSF samples were assessed for cell count, protein content and the presence of trypanosomes, for which in conjunction with the neurological signs, positive evidence for CNS involvement was according to the conventional criteria of protein concentration $\geqslant 0{\cdot}4$ g/l and/or white blood cell count $\geqslant 4 \times 10^6$/l and/or presence of trypanosomes. Patients were treated with MelB (Arsobal; Rhone-Poulenc) or a combination of pentamidine dimesylate (Rhone Poulenc) and Arsobal, and re-tested 6 weeks after treatment, when the symptoms had disappeared. Serum and CSF samples, collected at time of evaluation were transferred to endotoxin-free vacuum collection tubes (Kabi Diagnostica, Sweden) before shipment to the UK also as previously described (Pentreath *et al*. 1996).

EndoCAb measurement

Anti-endotoxin antibodies were detected by a modification of the ELISA technique developed by Barclay and Scott (see Barclay & Scott, 1987; Scott & Barclay, 1987). An equimolar mixture of incomplete-core LPS from 4 Gram-negative bacteria (*E. coli*, *S. tryphimurium*, *K. aerogenes* and *P. aeruginosa*), each complexed with polymyxin B, was coated on polystyrene microplates chosen for optimal EndoCAb characteristics. The polymyxin B enables both binding of endotoxins to the plate and their interaction with the antibodies. The inner core of each LPS was intact. The microplate strips were incubated with 100 μl buffer dilutions in duplicate of a standard and serum and CSF samples at 37 °C for 60 min and the wells washed 3 times with 0·1 M phosphate buffer, pH 7·4, containing 0·138 M NaCl, 0·1 % Tween 20 and 0·05 % sodium azide. Bound antibodies were detected by adding anti-human IgG antibodies conjugated with alkaline phosphatase ('Zymed' Cambridge Bioscience), incubating at 37 °C for 60 min, washing 3 times with rinse buffer, then 5 times with distilled water, and then incubating for 60 min at room temperature with alkaline phosphatase substrate (PNPP, Sigma). The absorbances were read at 405 nm, and measured against the standard curve.

EndoCAb values were expressed in median units (MU) per ml which relate the amounts to the median value (100 MU/ml) determined in a study of over 1000 healthy adults (see Barclay, 1995). From this and other clinical studies low and high EndoCAb values are < 35 MU/ml and > 250 MU/ml respectively (10th and 90th percentiles of normal adult range).

Endotoxin measurement

The blood and CSF samples were centrifuged (180 ***g*** for 10 min); the supernatant fractions were heated (75 °C for 10 min) and the endotoxin activity evaluated by a chromogenic modification of the *Limulus* amoebocyte lysate (LAL) test (Coatest, Chromogenix Ltd (Pentreath *et al*. 1996).

Statistics

EndoCAb and endotoxin levels in the serum and CSF of control and infected patients were assessed by Student's *t*-test. The relationship of the levels of the substances in the different fluid compartments were studied by correlation-regression analysis.

RESULTS

Patient symptoms

Following treatment the patient's symptoms and CSF criteria rapidly improved (normally within a few days after commencement), with marked reductions in protein content, leucocyte count and the elimination of trypanosomes.

EndoCAb and endotoxin in plasma and CSF of controls

The mean ($\pm$S.E.M.) EndoCAb level in the plasma of the 9 uninfected individuals was $173 \pm 17{\cdot}6$ MU/ml (range 90–250 MU/ml), in the CSF was $150 \pm 23{\cdot}3$ MU/ml (range 70–280 MU/ml). Mean endotoxin level in plasma was $10{\cdot}5 \pm 1{\cdot}1$ pg/ml (range 6·5–18·0 pg/ml), in CSF was $6{\cdot}6 \pm 0{\cdot}6$ pg/ml (range 5·0–8·5 pg/ml; Table 1, Fig. 1.).

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Table 1. EndoCAb and endotoxin in blood and CSF of control individuals and late-stage sleeping sickness patients

(EndoCAb values in MU/ml, endotoxin values in pg/ml, all values ± S.E.M. Pre, pre-treatment. Post, post-treatment.)

	Control		Patient			
			Serum		CSF	
	Serum	CSF	Pre	Post	Pre	Post
N	9	9	35	35	35	35
Mean EndoCAb (S.E.M.)	173 (17·6)	150 (23·3)	286 (23·5)	285 (25·9)	226 (15·8)	193 (15·0)
Range	90–250	70–280	100–650	100–690	90–415	90–410
Comparison of means difference	—	—	113	112	76	43
P	—	—	< 0·001	< 0·001	< 0·001	< 0·001
Mean endotoxin (S.E.M.)	10·5 (1·1)	6·6 (0·6)	54·5 (1·3)	28·1 (0·7)	51·9 (1·3)	26·8 (0·6)
Range	6·5–18·0	5·0–8·5	19·0–101·0	16·5–84·0	19·0–92·0	16–62
Comparison of means difference	—	—	44·5	17·6	45·3	20·2
P	—	—	< 0·001	< 0·001	< 0·001	< 0·001

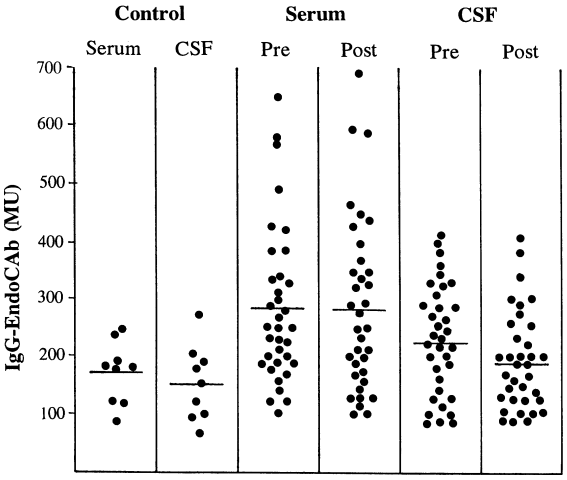


Fig. 1. IgG EndoCAb levels in the serum and CSF of controls and late-stage sleeping sickness patients before (pre) and 6 weeks after (post) chemotherapy. The bars are the mean values for each group.

EndoCAb and endotoxin in plasma of sleeping sickness patients

The mean (± S.E.M.) EndoCAb level in the plasma of the 35 patients was 286 ± 23·5 MU/ml (range 100–650 MU/ml), which is an elevation approximately 1·7 times over the control ($P < 0·001$; Table 1, Fig. 1). The mean level 6 weeks following treatment was 285 ± 25·9 MU/ml (range 100–690 MU/ml), which was not significantly different from the pre-treatment values. However, there was wide variation in individual responses, with 9 individuals showing post-treatment increases (range 20–60 MU/ml plasma), 6 showing decreases (range 20–90 MU/ml) and 20 with no significant change (> 20 MU/ml).

Endotoxin (± S.E.M.) values were 54·5 ± 1·3 pg/ml (range 19·0–101·0 pg/ml) pre-treatment, and 28·1 ± 0·7 pg/ml (range 16·5–84·0) post-treatment; these represent increases of 5·2 times (pre-treatment) and 2·7 (post-treatment) over the control values (for each $P < 0·001$). The mean post-treatment reduction was highly significant ($P < 0·001$) since all patient values apart from 2 individuals (who were unchanged) were reduced.

EndoCAb and endotoxin in CSF of sleeping sickness patients

The mean EndoCAb level in the patient's CSF (226 ± 15·8 MU/ml) was similar to plasma although, unlike plasma, no values exceeded 450 MU (CSF range 90–415 MU/ml). The mean increase was 1·5 times the control ($P < 0·001$; Table 1; Fig. 1). The mean post-treatment value was not significantly different (193 ± 15·0 MU/ml; range 90–410 MU/ml), and again there were different changes in individual values, with 7 patients showing post-treatment increases (range 20–70 MU/ml), 16 showing reductions (range 20–140 MU/ml) and 12 with no significant change (⩽ 20 MU/ml).

The mean CSF endotoxin was 51·9 ± 1·3 pg/ml (range 19·0–92·0 pg/ml), reduced to 26·8 ± 0·6 pg/ml (range 16–62 pg/ml) post-treatment. The mean patient value was increased 7·8 times over the control, and the post-treatment reduction to 4·1 times over control ($P < 0·001$ for both pre-treatment increases over control and post-treatment reduction over pre-treatment value).

Relationship between EndoCAb and endotoxin levels

The data were analysed for possible relationships between EndoCAb and endotoxin levels in the different fluid compartments and patient treatment and symptoms. Correlation-regression analysis showed a highly significant correlation between EndoCAb and endotoxin levels in the patient blood

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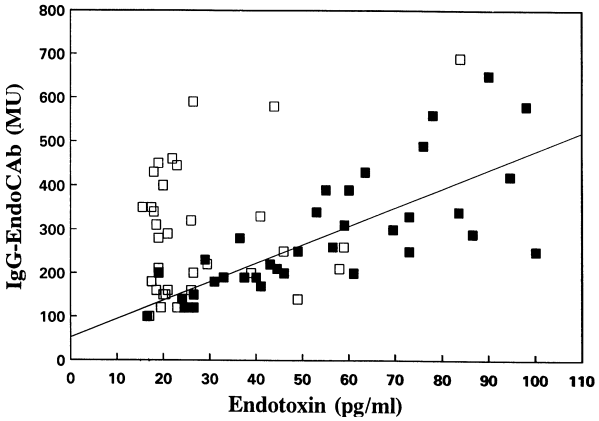


Fig. 2. Correlation between IgG EndoCAb and endotoxin levels in the serum of patients before (■) and after (□) chemotherapy. The regression line is for the pre-treatment values ($r = 0{\cdot}78$; $P < 0{\cdot}001$). There was no significant correlation for the post-treatment values, because the reductions in endotoxin levels were not associated with changes in IgG levels.

before treatment ($r = 0{\cdot}78$, $P < 0{\cdot}001$; Fig. 2), but not after treatment. This was directly associated with the significant reductions in endotoxin, but not EndoCAb, following chemotherapy. There was no significant correlation between EndoCAb levels and endotoxin levels in the CSF before or after treatment. There were also no correlations between EndoCAb levels in blood and CSF either before or after chemotherapy, which was surprising in view of the high degree of correlation in the endotoxin levels in the 2 compartments described previously (Pentreath *et al*. 1996). No correlations were found between the EndoCAb levels in blood or CSF with the patients' age, sex or other recorded symptoms or diagnostic criteria (including blood cell counts, protein levels and presence of trypanosomes).

DISCUSSION

The findings extend our earlier reports on the endotoxin involvement in trypanosomiasis in several ways. First, they provide direct evidence that Gram-negative bacteria are the principal source of the raised endotoxins detected in those studies (Pentreath *et al*. 1996). These previous measurements were made with the *Limulus* amoebocyte lysate (LAL) test, which can be prone to errors despite extensive precautions. The present ELISA methodology detected antibodies against the intact core/LPS regions of several gut bacteria, thus confirming our suggestions regarding the likely origin of the endotoxin. Although endotoxin-like activity is detectable by the LAL test in disrupted *T. b. brucei* (Alafiatayo *et al*. 1993), we have no evidence of this possibility for the human *T. b. gambiense* form. We also have no evidence that antibodies against components of any type of trypanosomes can cross-react with the core LPS from the Gram-negative bacteria used in the EndoCAb assay, and further experiments are required to test this. It must also be borne in mind that the measurements were limited to IgG class levels. In trypanosomiasis both IgG and IgM become elevated, but subsequently become suppressed. The extent of the remaining IgM response in particular, correlates with parasite virulence (Assoku, Hazlett & Tizard, 1979; Sacks *et al*. 1980). Although *T. b. gambiense* is predominantly a parasite of the extracellular spaces in tissues, and the IgG levels are very likely a good marker of chronic parasite exposure, further experiments are also necessary to evaluate IgM and IgA levels, and their variations during the infection, using the EndoCAb test system.

Both IgG and IgM EndoCAb levels become rapidly depleted during acute septicaemia from different sources of sepsis. However, the levels of IgG EndoCAb increase in situations of chronic exposure to endotoxin such as occurs in several forms of inflammatory bowel disease, urinary tract infections with renal stones and obstructive jaundice (summarized by Barclay, 1995). The present study demonstrates a striking correlation between EndoCAb and endotoxin levels in blood, which is consistent with a chronic exposure to endotoxin in this disease. The correlation between the levels of these substances in CSF, however, was weak. Furthermore, in contrast to the levels of endotoxin (Pentreath *et al*. 1996), there were no correlations between EndoCAb levels in CSF and blood. The CSF pool is thus behaving relatively independently of the blood with respect to the antibodies to endotoxin. Antibodies could reach the CSF via a damaged choroid or blood-brain barrier (BBB) both of which are known to occur in animal models of the disease (Pentreath, Baugh & Lavin, 1994; Philip *et al*. 1994). In a previous study on patients with late-stage disease symptoms Lambert, Berney & Kazyumba (1981) found elevated levels of immune complexes in sera and CSF, but the levels did not correlate in the 2 compartments. They suggested that the immune complexes were formed within the brains of patients. The present findings are consistent with their suggestion. At present the most appropriate conclusion is that the elevated endotoxins enter CSF via the damaged choroid and/or BBB, with subsequent generation of antibodies from infiltrated lymphocytes. Other information on the occurrence or sources of endotoxin antibodies in CSF which could shed light on the situation in trypanosomiasis is apparently not yet available.

The possible sources of the elevated endotoxin and some potential important implications for the disease were discussed in a previous report (Pentreath *et al*. 1996). The present work describing the specificity for Gram-negative bacteria and chronicity of exposure also argues strongly for further studies to determine the roles in the disease pathology.

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