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Expression of the B cell repertoire and autoantibodies in human African trypanosomiasis

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SUMMARY

The relative importance of polyclonal B cell activation has been studied in relation to the development of autoantibodies in human African trypanosomiasis. In 34 patients investigated before specific treatment a broad expression of the B cell repertoire was observed including the production of anti-hapten (FITC, Penicillin, Phosphorylcholin) antibodies, of high levels of antibodies against some heterologous protein antigens (ovalbumin and tetanus toxoid) and of autoantibodies. Anti-ssDNA antibodies were detected in 84% of the patients and anti-IgG rheumatoid factors in 88%. Anti-striated muscle and anti-smooth muscle antibodies were also observed in 57 and 63% of the patients. Correlation analysis indicated that the formation of anti-DNA antibodies is associated with polyclonal B cell activation but probably depends on an additional B cell stimulation by released DNA or cross-reacting antigens. Anti-immunoglobulin antibodies are closely correlated with polyclonal B cell activation and their production is likely to reflect the high frequency of anti-IgG B cell precursors in the normal human B cell repertoire.

The significance of these observations in relation to the pathological expression of trypanosomiasis should be particularly considered in the generation of immune complexes either in circulating blood or locally at the sites of parasite destruction.

Keywords trypanosomiasis polyclonal B cell activation autoantibodies DNA

INTRODUCTION

A classical immunologic feature of African trypanosomiasis is a production of high amounts of immunoglobulins. Antibodies with specificities against trypanosome antigens may represent a minor proportion of these immunoglobulins (Houba, Brown & Allison, 1969; Freeman *et al.*, 1970) and it has been proposed that the increase in immunoglobulin production could reflect a nonspecific polyclonal B-lymphocyte activation (Greenwood, 1974). However, it has been reported that most serum immunoglobulins could be absorbed using variable surface glycoproteins from the successive variants of the infecting trypanosome, in *Trypanosoma congolense* infected cattle (Musoke *et al.*, 1981). In mice infected with *Trypanosoma brucei brucei* (*T. brucei*), it has been shown that there is a rapid increase in the number of IgM antibody-producing cells in the spleen (Hudson *et al.*, 1976). In parallel, there is a marked increase in the number of plaque-forming cells to trinitrophenol (TNP), fluoroscein isothiocyanate (FITC) or sheep red blood cells (RBC) (Kobayakawa *et al.*, 1979). Autoimmune responses to DNA, red blood cells and thymocyte antigens were also observed in

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these mice and the kinetics of the formation of autoantibodies was parallel to the development of polyclonal B cell activation.

The purpose of this investigation was to evaluate the relative importance of antigen-non-specific activation of B cell clones in the induction of auto-antibodies in human patients with *T. brucei gambiense* infection. Thus the incidence of anti-hapten and anti-heterologous protein antibodies, as markers of polyclonal B cell activation, was studied in correlation with the incidence of autoantibodies. A follow up study of these parameters was done during and after treatment with melarsoprol.

MATERIALS AND METHODS

Patients. Serum samples from 34 patients with African trypanosomiasis were collected in Zaire during the regular routine activities of the 'Centre du Contrôle de la Trypanosomiase'. The clinical diagnosis was confirmed by the microscopic demonstration of trypanosomes in blood, in lymph node aspiration fluid or in cerebro-spinal fluid (CSF). All these patients had anti-trypanosome antibodies in serum. Three different stages of the disease were considered: patients at stage I present haemolymphatic manifestations of the infection (enlarged lymph nodes, trypanosomes in blood, normal pattern of cerebrospinal fluid, no clinical sign of cerebral involvement); stage II is similar to stage I but trypanosomes appear in the CSF; stage III is characterized by neurological manifestations ('sleeping sickness') and abnormal CSF (trypanosomes, increased cell and protein content). In our study, three patients were in stage I, three in stage II and the others in stage III. Serum samples were also obtained during treatment and after treatment with melarsoprol (Arsobal, Specia, Paris, France). Blood was drawn from patients, allowed to clot, centrifuged and frozen, stored and shipped in liquid nitrogen. Control serum samples were obtained in Kinshasa from age-matched normal Zairian individuals and from hospitalized Zairian patients suffering from schistosomiasis (8), amebiasis (2), ascariasis (2), hookworms (2), lung cancer (1) and tuberculosis (2). Another group of control sera was obtained from 20 age-matched normal blood donors in Geneva.

Quantitation of anti-trypanosome antibodies. Trypanosoma brucei were obtained from LUMP 227 (London University Medical Protozoology) after passage in irradiated mice. Trypanosomes were separated from red blood cells on a DEAE 52 column according to Lanham & Godfrey (1970). Smears were prepared, dried and fixed with ethanol. Serial dilutions of each serum were then tested for antibodies by indirect immunofluorescence using anti-IgG and anti-IgM conjugates (Nordic Laboratories, The Netherlands).

Preparation of hapten-protein conjugates. Bovine serum albumin (BSA) or Egg albumin (OVA) were coupled to the 2,4 dinitrophenyl (DNP) haptenic group by reacting 10 mg of 2,4 dinitrobenzene-sulfonate (Eastman Kodak C., Rochester, NY, USA) with 30 mg of BSA (DNP-BSA) or OVA (DNP-OVA) according to the method of Eisen, Belman and Carsten (1953). The ratio DNP groups/molecule of protein was 30–35.

Penicillin G (potassium salt, 1×10^6 units, Squibb S.p.A., Roma, Italy) dissolved in 0·5 M K_2CO_3 was conjugated with 10 mg BSA (PEN-BSA) according to Little and Eisen (1967).

p-Diazonium phenyl phosphorylcholine (DPPC) was prepared according to Chesebro and Metzger (1972) and DPPC-bovine serum albumin (PC-BSA) conjugates were prepared by reacting $3{\cdot}7 \times 10^{-6}$ M DPPC with $5{\cdot}0 \times 10^{-6}$ M BSA according to Claflin, Lieberman and Davie (1974). The degree of coupling was 11·8 mol per mol of PC-BSA.

Fluorescein isothiocyanate isomer I (6 mg FITC, Becton Dickinson, Cockeysville, MO, USA) was dissolved in carbonate-bicarbonate buffer 0·05 M pH 9·4, mixed with 20 mg BSA and incubated overnight under gentle mixing at 4°C. Protein unbound FITC was eliminated by extensive dialysis against carbonate-bicarbonate buffer. The FITC/BSA ratio was always over 10.

Tetanus toxoid. Tetanus toxoid was kindly provided by the Wellcome Laboratories (Beckenham, UK) radiolabelled and diluted to 200 ng/ml for testing.

Radiolabelling procedures. All hapten-protein complexes or proteins were radiolabelled with ^{125}I-Na (Radiochemical Center, Amersham, UK) by the Chloramine-T method (McConahey & Dixon, 1966).

Radioimmunological methods and enzyme-immunoassays. Radioimmunoassays for anti-DNP-BSA, anti-PC-BSA, anti-FITC-BSA, anti-PEN-BSA, anti-BSA, anti-Ovalbumin, anti-DNP-OVA and anti-tetanus-toxoid antibodies were done, using a modified Farr-radioimmunoassay. In brief, serum samples were diluted 1/10 or 1/125 in borate buffer, heat inactivated at 56°C for 30 min; 0·1 ml of diluted serum were mixed with 0·1 ml of the radiolabelled antigen (100 ng/ml for PC-BSA, 200 ng/ml for tetanus toxoid and 1 μg/ml for the other antigens and with 0·05 ml of 0·125% sodium dodecylsulfate (SDS), then were left for incubation overnight at 4°C. One ml of 41·25% saturated ammonium sulfate (SAS) in borate buffer (final concentration = 33·3%) was added to each tube and incubated 30 min at 4°C before centrifugation at 1,500 *g* at 4°C. The supernatant was discarded, and and the precipitate washed with 1 ml 33·3% SAS. The tubes were centrifuged and the radioactivity of the precipitates was measured. Results were expressed as the percentage of precipitation. All tests were done in duplicate.

A solid phase radioimmunoassay was used for the detection of anti-KLH antibodies. Polypropylene microtubes were coated with 0·1 ml KLH (100 μg/ml) (keyhole limpet haemocyanin, Calbiochem, San Diego, CA, USA) left 3 h at 37°C and overnight at 4°C. The tubes were washed three times with VBS-Tween (0·25%) then 0·1 ml 1:20 heat inactivated serum was added and incubated for 2 h at room temperature followed by three more washings with VBS-Tween. 0·1 ml rabbit anti-human IgG or rabbit anti-human IgM (Hoechst-Pharma AG., Zürich, Switzerland) diluted 1:50 in VBS, was added and incubated 30 min at room temperature, followed by five washes with VBS-Tween. 0·1 ml ^{125}I-labelled protein A (0·5 μg/ml) was added and incubated for 4 h at 4°C. The tubes were washed three times in VBS-Tween and the radioactivity remaining on the tubes was counted. The results were expressed as per cent protein A bound.

A similar solid phase assay was used for the measurement of anti-IgG antibodies. The coating was done with rabbit or human IgG and the quantitation was achieved with enzyme-conjugated anti-human IgM antibodies.

Anti-ssDNA and anti-dsDNA antibodies were measured using a modified Farr DNA-binding RIA (Izui, Lambert & Miescher 1976).

Quantitation of serum immunoglobulins. IgG, IgM and IgA were measured by radial immunodiffusion. IgE levels were determined by radioimmunoassay (Pharmacia, Uppsala, Sweden).

Autoantibodies against tissue specific human antigens. Antibodies to thyroglobulin, thyroid microsomes, gastric parietal cells, salivary and adrenal glands as well as to striated and smooth muscles, were detected by indirect immunofluorescence using human tissues as substrate. Antibodies against intrinsic factor were detected by a radioimmunoassay using ^{57}Co-cyanocobalamin (Radiochemical Center, Amersham, UK). Anti-human red cells antibodies were detected by classical immunohaematological techniques.

Statistical analysis. Comparison between patients and control groups were done using a Wilcoxon rank test. Correlation analysis were performed using the Spearman ranking test.

RESULTS

Immunoglobulin levels

As expected, high levels of serum immunoglobulins were observed in patients with trypanosomiasis at all stages of the disease (Table 1). The levels of IgG did differ significantly from those observed in normal Zairian controls ($P < 0{\cdot}001$). There was a six-fold increase of the IgM levels which differed significantly from both normal and hospitalized controls. There was also a slight increase of IgA levels and of IgE levels. IgD was not detectable in the patients' sera.

Polyclonal stimulation of antibody synthesis during African trypanosomiasis

Antibodies to various haptens were measured by radioimmunoassay in all patients (Fig. 1). Significantly high levels of antibodies were observed against FITC, Penicillin and Phosphorylcholine as compared to African patients with other parasite diseases and normal African controls ($P < 0{\cdot}001$). Anti-DNP antibodies were increased only as compared to other hospitalized patients. All these values were much higher than in normal European controls (data not shown). It should be

Table 1. Immunoglobulin levels in trypanosomiasis

		IgG (mg/ml)	IgM (mg/ml)	IgA (mg/ml)	IgE (units/ml)
Trypanomsomiasis	($n=34$)‡	34·6*±11·5†	12·4±7·4†	3·3±2·3	1188±1089
Other hospitalized patients	($n=10$)	31·1±13·8	4·2±3·3	2·6±1·4	3104±800
Normal Zairian controls	($n=12$)	16·3±4·9	1·8±0·6	2·0±0·7	508±377
Normal European controls	($n=20$)	11·3±2·7	1·6±0·4	2·2±1·0	180±10

* Mean ± 1 s.d.
† $P<0·001$, significance as compared to normal Zairian controls, (Wilcoxon).
‡ pretreatment samples.

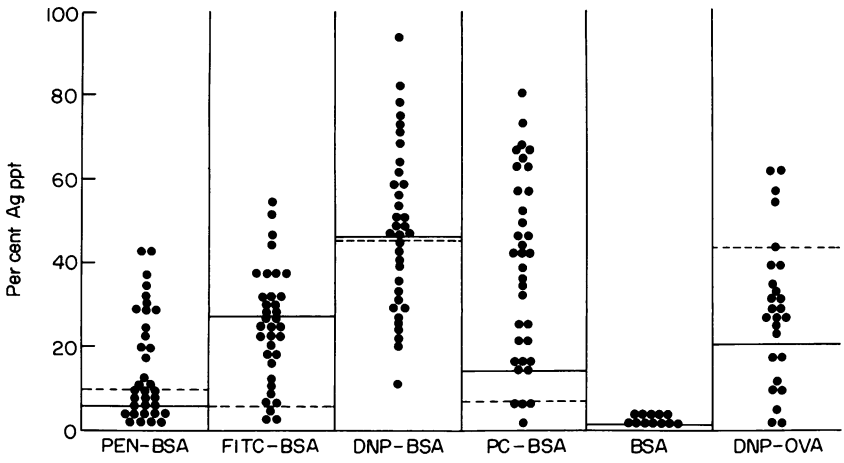

Fig. 1. Levels of anti-hapten antibodies in patients with trypanosomiasis (pretreatment samples). The results are expressed in percent of labelled antigen precipitated. For each system the interrupted line (– – –) represents the limit of the 90th percentile in normal Zairian controls and the continuous line (——) in other hospitalized Zairian patients.

pointed out that none of the patients exhibited anti-BSA antibodies although their serum reacted with hapten-conjugated BSA. In addition anti-DNP antibodies were also detected using ovalbumin instead of BSA as a carrier for DNP. The binding of DNP-BSA or of DNP-OVA was not inhibited in presence of either cold BSA or cold ovalbumin (10 mg/ml).

Anti-SRBC and anti-protein antibodies. In most patients with trypanosomiasis there was a high level of antibodies against ovalbumin and tetanus toxoid as compared to normal Zairian controls. The levels of anti-SRBC and of anti-KLH antibodies were high but did not differ significantly from the control population (Table 2).

Anti-DNA, anti-IgG and other autoantibodies. Antibodies to ssDNA were detected in 84% of the patients with trypanosomiasis (mean level of 17·1±7·7%) as compared with other hospitalized African patients (6·3±2·7%) normal Zairian controls (12·1±4·3%) or European controls (9·8±4·7%) (Fig. 2). In those patients, antibodies to DS-DNA were not detectable.

Rheumatoid factor-like (RF) anti-immunoglobulin antibodies were detected by solid phase RIA. IgM anti-IgG antibodies were found in 30/34 patients with trypanosomiasis. The mean levels were 485±267 ng anti-IgM bound/ml serum and 176±116 ng anti-IgM bound/ml serum in normal

Table 2. Anti-protein antibodies in trypanosomiasis

		SRBC (log. 2)	OVA % ppt	TeT. ToX. % ppt	KLH IgG Protein A bound (%)	KLH IgM Protein A bound (%)
Trypanosomiasis	($n=34$)§	5·8*±2·2	19·6±7·2†	66·3±14·6‡	32·6±10·2	28·2±9·9
Other hospitalized patients	($n=10$)	4·8±2·6	9·9±5·2	50·4±15·5	19·4±11·2	24·9±10·7
Normal Zairian controls	($n=12$)	5·1±0·5	12·3±10·5	21·7±19·6	30·5±16·8	23·7±19·8
Normal European controls	($n=20$)	2·1±1·3	12·2±2·7	12·6±1·7	12·1±7·3	12·7±3·3

* Mean ± 1 SD

† $P<0·01$; ‡ $P<0·001$; significance as compared to normal Zairian controls, (Wilcoxon).

§ Pretreatment samples.

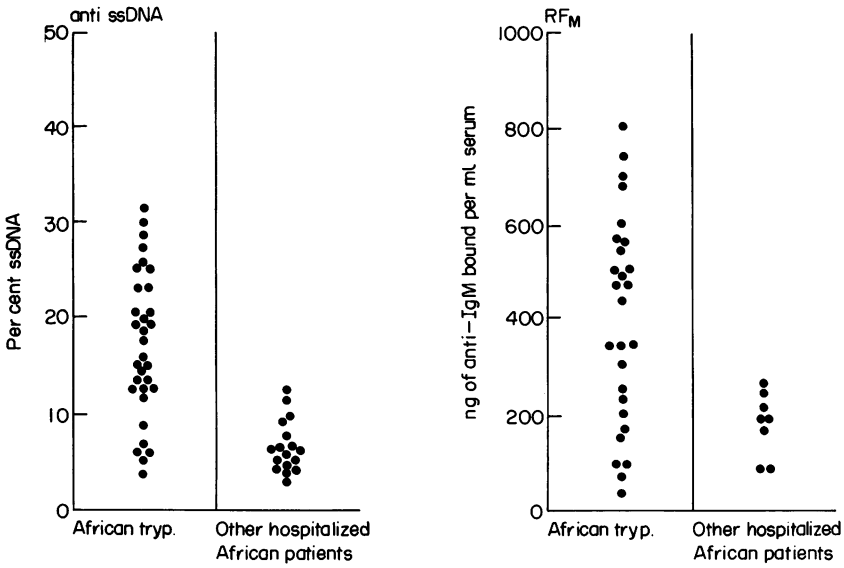

Fig. 2. Levels of anti-ssDNA antibodies and of IgM anti-IgG antibodies (RF-M) in patients with trypanosomiasis (pretreatment samples) as compared with other hospitalized African patients. The levels of anti-DNA antibodies are expressed in per cent DNA precipitated in a Farr assay. The levels of IgM anti-IgG antibodies (RF) are expressed as the amount of anti-IgM probe which, in a second step, reacted with IgM antibodies bound to solid-phase IgG.

Zairian controls. There was no increase in the level of IgG anti-IgG antibodies. Autoantibodies against tissue specific antigens were searched for by indirect immunofluorescence or by RIA. There were no detectable antibodies against thyroglobulin, thyroid microsomes, intrinsic factor, gastric parietal cells, salivary glands nor against adrenal tissues. However, antibodies against striated muscle and against smooth muscle were detected in respectively 57% and 63%, of patients with trypanosomiasis, with titres of 1/20–1/160. Anti-smooth muscle antibodies were mostly of the IgM class while anti-striated muscle were found in both IgG and IgM classes. It should be noted that the

anti-striated muscle antibodies differed from those observed in myasthenia gravis. Indeed, they stained only narrow bands on the muscle fibres. Antinuclear antibodies (speckled type) were found at low titres in about half of the sera tested.

Cold agglutinins reacting with normal human red cells (group O) were found in 3/19 sera at titres 1/8 to 1/64.

Characterization of anti-haptens and anti-DNA antibodies

Five serum samples from patients with trypanosomiasis were fractionated by sucrose gradient ultracentrifugation and the various fractions were analysed for their capacity to bind DNP-BSA, PC-BSA, FITC-BSA and DNA. The binding of DNP, PC and FITC was mostly limited to the 7S fractions with occasional binding in the 19S fractions. Inversely, the binding of DNA was mostly observed in the 19S fraction (Fig. 3). After chromatography on DEAE cellulose, the IgG fractions from seven patients were obtained and tested for their reactivity with haptens and with DNA as compared to IgG isolated from normal serum. Antibodies to PC-BSA and to DNP-BSA were detected with high activity in the 7S IgG fractions tested, but not in the control IgG fractions. Anti-FITC-BSA were found in 3/7 IgG fractions but anti-ssDNA-antibodies were detected only in 1/7 IgG fractions. One of the IgG fractions with a high DNP-binding activity was digested with pepsin and $F(ab)_2$ fragments were tested for their activity. There was persistence of DNP-binding after pepsin digestion.

The effect of deoxyribonuclease (DNase) on the DNA-binding activity was tested on 9 serum samples: it did not increase the DNA-binding activity. Thus, there was no direct evidence of

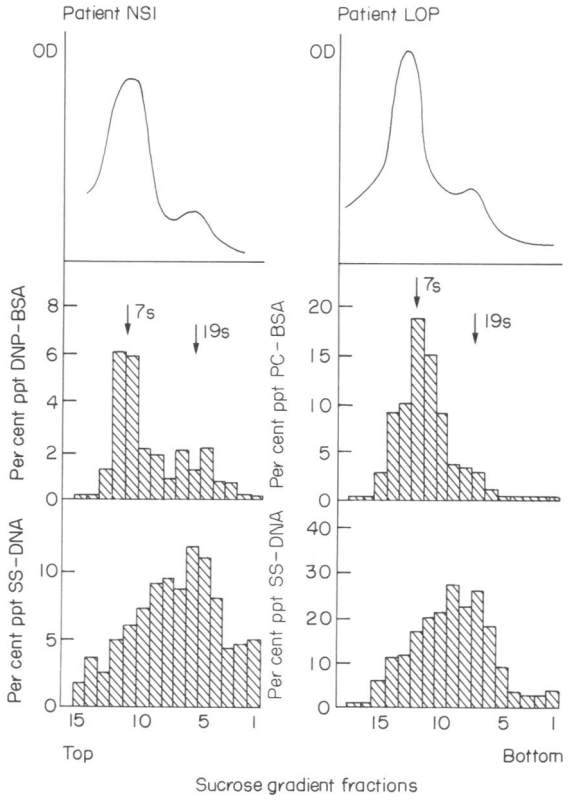

Fig. 3. Profile of anti-ssDNA, anti-DNP-BSA and anti-PC-BSA antibody activities in sera from patients with trypanosomiasis fractionated by ultracentrifugation on sucrose gradients. The top part represents the optical density curves obtained after centrifugation and the arrows indicate the positions of the 7S and 19S markers.

circulating DNA-anti-DNA complexes. The binding of hapten-protein conjugates by patients' serum samples was partially inhibited in presence of an excess of cold conjugate or of free hapten. For example, on 10 samples, the mean binding of PEN-BSA decreased from 28·5 ± 8·9 to 18·9 ± 7·4 after addition of 100 times cold PEN-BSA and to 21·00 ± 9·3 after addition of Penicillin (200 μg/ml).

Correlation between polyclonal B-cell activation and anti-trypanosome antibodies

The level of antibodies to SRBC, PC-BSA and DNP-BSA was tested in six trypanosomiasis serum samples with or without previous absorption on heat-killed *Trypanosoma brucei brucei*. There was no significant change in the level of activity. In addition, there was no significant correlation between the serum level of IgM or IgG and the titres of anti-trypanosome antibodies.

Analysis of correlations between autoantibodies and immunoglobulin levels, anti-hapten or anti-protein antibodies

Significant correlations ($P < 0{\cdot}005$) between the levels of IgM and those of rheumatoid factor (Fig. 4) were observed. A similar correlation was seen between IgG levels and anti-hapten antibodies (DNP, FITC). Furthermore, there was significant correlations between the levels of each type of anti-hapten antibodies as well as between those antibodies and anti-IgG antibodies.

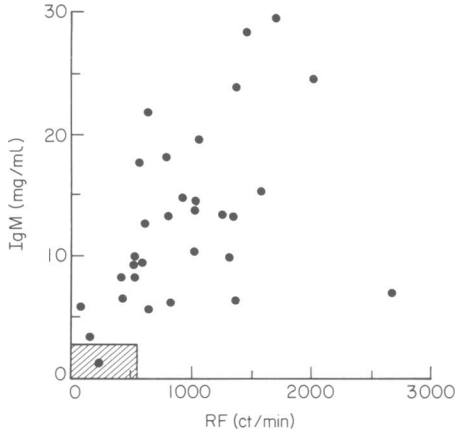

Fig. 4. Correlation between IgM-anti-IgG (RF) and IgM levels in patients with African trypanosomiasis (pretreatment samples). $R = 0{\cdot}59$; $P < 0{\cdot}005$.

Follow-up studies

Seven patients were studied 0, 2, 4, 8, 15 and 22 days during treatment with Melarsoprol. During that short period, there was no significant variation of IgG levels while there was a significant decrease of IgM levels in 4/7 patients. In these patients, there was also a significant decrease in the levels of anti-ss-DNA, anti-PC-BSA and anti-DNP-BSA.

DISCUSSION

Our study demonstrates that the polyclonal B cell activation characterizing human African trypanosomiasis is associated with a broad expression of the B cell repertoire including anti-hapten, anti-heterologous protein antigens and a variety of autoantibodies. Some of these features are likely to reflect an antigen-non-specific B cell activation. This would be particularly the case for anti-hapten antibodies. Indeed, the immune response against trypanosome antigens did not appear responsible for the induction of anti-DNP antibodies since those antibodies could not be absorbed by heat-killed trypanosomes. The significant correlations between IgG levels and anti-DNP or anti-

FITC levels is also suggestive that the presence of anti-hapten antibodies reflects a non-specific polyclonal B cell activation. However, one cannot entirely exclude that some epitopes of variant antigens would cross-react with haptenic determinants. It is likely that the relatively high background values observed in some other hospitalized patients and in some normal Zairian controls are related to the high prevalence of other parasitic infections such as malaria.

The increased production of some anti-heterologous protein antigens obviously may reflect the activation of already primed B cells as suggested by the high levels of anti-tetanus toxoid antibodies observed in this population. The formation of autoantibodies in patients with African trypanosomiasis is probably also dependent on polyclonal B cell activation but it may reflect an additional specific stimulation by autoantigens or by cross-reacting antigens. Thus, although most patients exhibited anti-DNA antibodies there was no correlation between the total levels of immunoglobulin and DNA-binding activities. It is likely that large amounts of DNA are released from dying parasites or from altered tissues. In addition, monoclonal anti-DNA antibodies derived from autoimmune mice were recently shown to cross-react with bacterial phospholipids (Carroll *et al.*, 1985) and such cross-reaction may exist with trypanosome membrane constituents. In this situation, polyclonal B cell activation would act as a complementary signal for antigen-specific B cells and would alleviate the requirement of helper T cells.

The formation of IgM anti-IgG antibodies in most patients with trypanosomiasis was more closely associated with other features of polyclonal B cell activation. Indeed, there were significant correlations between levels of RF-activity, serum IgM and anti-hapten antibodies. In man, the development of rheumatoid factors has been observed in several situations characterized by polyclonal B cell activation such as infectious mononucleosis or visceral leishmaniasis (Galvao Castro *et al.*, 1984). Such RF have also been observed in mice after induction of polyclonal B cell activation by injection of bacterial lipopolysaccharides (Izui, Eisenberg & Dixon, 1979; Louis & Lambert, 1979). It appears now that B cell precursors for Fc-specific anti-IgG antibody-producing cells exist with a relatively high frequency in normal individuals and that all stimulations leading to a significant expression of the B cell repertoire would lead to the production of RF.

The mechanisms involved in the triggering of polyclonal B cell activation during trypanosomiasis have been studied in mice. T cells did not appear to play an important role in this activation since it was seen with a comparable intensity in infected athymic nu/nu mice (Kobayakawa *et al.*, 1979). The mitogenic stimuli can be derived from the parasites as suggested by the in-vitro demonstration of a mitogenic effect of parasite extracts (Esuruoso, 1976). It should be pointed out that all B cells do not respond equally to in-vivo polyclonal stimulation. Differences in the spectrum of antibodies produced may be related to a simultaneous exposure to other reacting epitopes, to prior priming and expansion of some B cell clones, to differences in B cell sensitivity to mitogenic effects at the time of stimulation or, as in malaria, to the involvement of T cells in B cell activation.

The significance of polyclonal B cell activation and of the triggering of autoreactive B cells in trypanosomiasis should be considered in relation to the pathological expression of the infection. Some auto-antibodies, e.g. anti-DNA, can react with DNA released in tissue lesions from parasites or from host cells and amplify the inflammatory reaction through the local formation of immune complexes. This was suggested by the demonstration of anti-DNA antibodies in acid eluates from myocardial tissue in mice infected with *T. brucei brucei* (Galvao Castro *et al.*, 1978). Furthermore, anti-IgG antibodies appearing during trypanosomiasis were shown to react with human IgG and their involvement in the generation of circulating immune complexes is likely. Indeed, we have observed a close correlation between the levels of anti-IgG antibodies and of circulating immune complexes in patients with trypanosomiasis (Lambert, Berney & Kazyumba, 1981). In fact, it was demonstrated in animal models that all events leading to a broad expression of the B cell repertoire are associated with interactions between immunoglobulin molecules due to anti-Fc antibodies (Ramos-Niembro, Fournié & Lambert, 1982) or anti-idiotypic antibodies (Rose, Goldman & Lambert, 1982). Therefore, IgG-anti-IgG complexes resulting from polyclonal activation may take part in the vascular pathology associated with trypanosomiasis.

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