Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 40 (1983)
Heft: 4

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DOI: https://doi.org/10.5169/seals-313139

Acta Tropica *40*, 321–329 (1983)

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Relevance of autoantigens to autoimmunity in African trypanosomiasis: study of DNA and thyroglobulin antibodies

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Summary

In order to investigate whether the autoantibody production in the course of African trypanosomiasis is the result of a generalized polyclonal activation or if it is a specific (antigen-dependent) phenomenon we looked for the presence of autoantibodies directed against autoantigens likely to be released (DNA) or unlikely to be released (thyroglobulin) into the circulation during human *T. gambiense* infection. Sera from 21 patients with African trypanosomiasis were screened for anti-native DNA or anti-denaturated DNA antibodies by a Farr DNA binding radioimmunoassay. In addition, antibodies to native DNA were also assessed by indirect immunofluorescent assays using *Crithidia luciliae* or rat liver sections as substrates. Anti-thyroglobulin antibodies were studied by the passive hemagglutination technique. IgM levels and fluorescent anti-trypanosomal antibodies (FATA) were concomitantly evaluated. The denaturated DNA binding capacity was significantly higher in the sera from trypanosomiasis patients than in sera from healthy blood donors. These capacities were greater in patients with high IgM levels and high FATA titres. Anti-native DNA or anti-thyroglobulin antibodies were not detected. This preferential formation of anti-denatured DNA antibodies suggests a specific antigen-dependent) activation of autoreactive cells rather than a generalized polyclonal activation.

Key words: *Trypanosoma gambiense;* autoimmunity; anti-DNA antibodies; anti-thyroglobulin antibodies; polyclonal B-cell activation.

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Introduction

Natural or experimental African trypanosomiasis is accompanied by the host producing large amounts of immunoglobulins (Ig) and the formation of antibodies directed against several hetero- and autoantigens. Polyclonal B-cell activators (PBA) such as bacterial lipopolysaccharides (LPS) induce cell proliferation, differentiation and synthesis of antibodies of different specificities including autoantibodies (AAb) (reviewed by Morrison and Ryan, 1979). Since trypanosomes too are endowed with polyclonal B-cell activating properties (Esuoroso, 1976; Assoku et al., 1979) it is possible that the mechanisms responsible for the AAb production observed during African Trypanosomiasis (reviewed by Wolga et al., 1981) are similar to those involved in the development of AAb induced by PBA.

Natural human African trypanosomiasis could therefore represent a model for the study of the modality of induction of AAb by PBA. Indeed it is yet questionable whether PBA activates autoreactive cells (ARC) directly in a non-specific way, as proposed by Coutinho and Möller (1974), or if they act only as a second (non-specific) signal to cells receiving the antigenic (specific) signal. According to the former hypothesis activation of ARC by mitogens would be non-specific and therefore generalized, according to the second it would be dependent upon the presence of the specific antigen. In this case only the cells concomitantly exposed to immunogenic amounts of the autoantigen can be activated to AAb synthesis.

To clarify this point, we investigated the presence of antibodies (Ab) directed against autoantigens likely to be released (DNA) or unlikely to be released (thyroglobulin) during the course of African trypanosomiasis.

Materials and Methods

Serum samples. – Sera were obtained from 21 African and European patients with African trypanosomiasis who attended to the «Laboratoire Central de Parasitologie et Consultation des Maladies Tropicales» at Hôpital de la Pitié, between 1976 and 1981. Ages ranged from 8 to 54 years old; 16 males and 5 females were studied. The diagnosis of African trypanosomiasis *(T. gambiense)* was established on the basis of clinical, parasitological and/or serological features. Identification of the species was substantiated by geographical data.

Assays for antibodies to deoxyribonucleic acid (DNA). – Farr DNA binding radioimmunoassay. Antibodies to native or double-stranded (ds) DNA and denaturated or single-stranded (ss) DNA were assessed by a Farr ammonium sulphate assay using radioactive DNA (Wold et al., 1968). ^{125}I-dsDNA (Amersham Ltd) was used unaltered or heated at 100° C for 10 min and plunged immediately afterwards into an alcohol bath at −80° C to obtain heat-denaturated DNA (^{125}I-ssDNA). Sera were heat-inactivated at 56° C for 30 min prior to assay. The standard assay employed 50 µl of serum diluted at 1/10 in a borate buffer and 50 µl of ^{125}I-DNA. The mixture was incubated for 1 h at 37° C and overnight at 4° C followed by precipitation with an equal volume (100 µl) of 100% saturated ammonium sulphate (AS) (final AS concentration 50%). Tubes were then centrifuged at 4000 *g* for 15 min and the supernatants were discarded. The radioactivity of the precipitates was measured in a gamma counter for 120 sec. Results were expressed as percentages of precipitated

^{125}I-DNA. A control tube containing 50 μl of ^{125}I-DNA gave the amount of radioactivity introduced in the assay.

Immunofluorescent antibody test (IFAT) on Crithidia luciliae substrate. – Antibodies to dsDNA were also assessed by the IFAT using *Crithidia luciliae* as substrate as described by Aarden et al. (1975). Neat or 1/10 diluted serum were incubated with *C. luciliae* previously fixed in acetone. Fluorescein-conjugated polyvalent anti-human immunoglobulin (Institut Pasteur) was used at a 1/100 dilution. Appropriate positive and negative controls were included in each set of slides examined.

IFAT on rat liver sections. – Anti-nuclear antibodies (ANA) were studied by the IFAT using unfixed 4 μm rat liver sections as substrate as described by Roitt and Doniach (1966).

Anti-thyroglobulin antibody assay. – Anti-thyroglobulin antibodies were studied in 54 sera, obtained from the 21 trypanosomiasis patients, by the indirect haemagglutination technique using tanned turkey red blood cells sensitized to human thyroglobulin (thymune T test Wellcome).

IFAT for detection of anti-trypanosomal antibodies. – Antibodies to trypanosomal antigens were assayed by the classical indirect technique as described by Ambroise-Thomas (1969). Briefly, sera were diluted at 1/100 and incubated 30 min at 37° C with *T. equiperdum* antigen previously fixed for 10 min in acetone. After washing in phosphate buffered saline (PBS), the preparations were incubated as described above with commercial fluorescein conjugated polyvalent anti-human immunoglobulin serum (Institut Pasteur) diluted at 1/100 in a 1/50,000 Evans blue counterstain solution. Titres were determined by standard two-fold dilutions in PBS (pH 7.2) starting at 1/200.

Serum immunoglobulin M (IgM) concentration was estimated by the Mancini method of single radial immunodiffusion using "Hyland" immunoplates.

Statistical evaluation was performed by Student's t-tests for paired or non-paired data as appropriate.

Results

The mean percentage of precipitated ^{125}I-ssDNA was higher in the trypanosome-infected patients (48.9 ± 7.8%) than in 10 blood donors (24.5 ± 8.1%); this difference was statistically significant ($t = 7.9$, $p < 10^{-9}$). In addition, results obtained with sera from all but one trypanosome infected individual were higher than the maximum value (34.3%) observed in the control subjects (Fig. 1). No differences in the ssDNA binding capacity were observed between African (46.85 ± 7.3%) and Europeans (52.4 ± 8.6%) ($t = 1.48$), males (48.2 ± 8.3%) and females (52.2 ± 5.2%) and those above (49.2 ± 9%) and below 30 years of age (48.8 ± 4.4%).

The specificity of anti-DNA Ab for ssDNA was confirmed by the results obtained with different methods: the Farr DNA-binding radioimmunoassay using native (ds) or heat denaturated (ss) DNA, the IFAT performed on *Crithidia luciliae* antigen and the IFAT using rat liver sections as substrate.

Fig. 2 shows the percentage of ds and ss DNA bound by each one of 21 sera obtained from 18 patients (3 of them were collected at the time and after the acute phase of infection). Each serum could bind twice more ssDNA than dsDNA. The mean of the differences between the amount of ssDNA and dsDNA bound by each serum (24.8 ± 7.3%) was statistically significant ($t = 15.6$, $p < 10^{-9}$). In addition none of these sera gave positive reactions on *Crithidia luciliae* antigen or on rat liver sections.

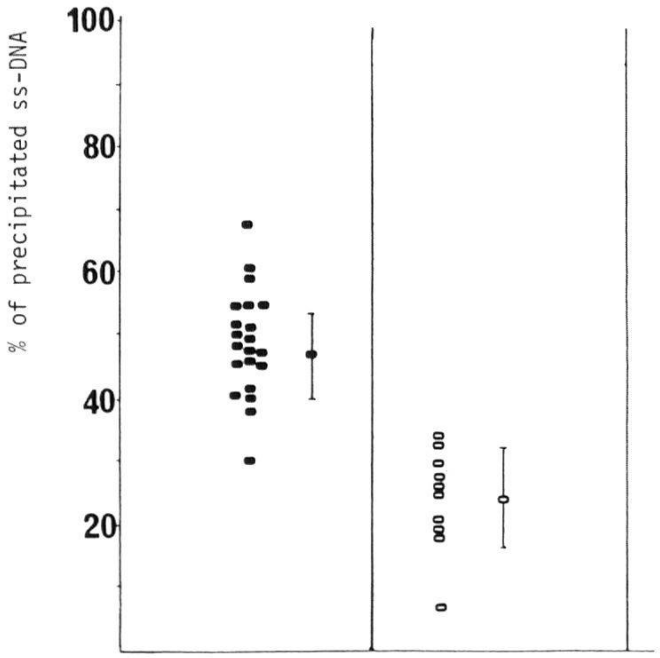

Fig. 1. ssDNA binding capacity in: (▪) sera from 21 *T. gambiense* infected subjects sera, (▫) sera from 10 healthy blood donors sera.

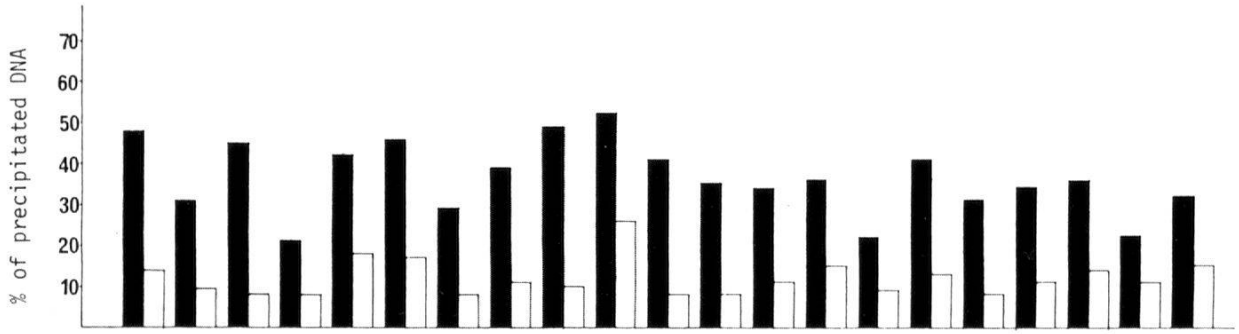

Fig. 2. Anti-ssDNA (■) and anti-dsDNA (□) activity in sera from 18 *T. gambiense* infected subjects.

Relationship between anti-ssDNA antibodies, anti-trypanosome fluorescent antibodies, and serum IgM

To investigate whether the production of AAb was correlated with either the specific response to trypanosomal antigens or with the non-specific response to parasite mitogens, we considered anti-trypanosomal antibody titres and serum IgM levels as markers of the specific and non-specific responses respectively.

No positive relationship was observed between the reciprocal titres of fluorescent anti-trypanosome Ab (FATA) and the percentage of precipitated ssDNA in the 31 sera studied ($r = 0.26$). However, the mean percentage of precipitated ssDNA was significantly higher ($t = 2.18$, $p<0.03$) in the group characterized by "high" titres of FATA (ranging from 1/1600 and 1/6400) ($47.9 \pm 9.3\%$) than in that of "low" FATA titres (ranging from 1/200 to 1/800)

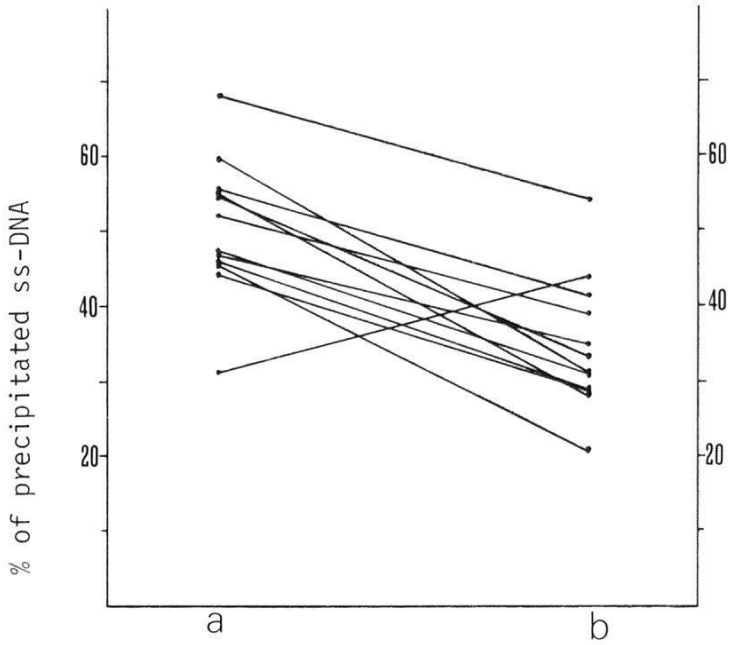

Fig. 3. Anti-ssDNA activity in sera from 12 *T. gambiense* infected subjects collected on the onset of the disease (a) and after treatment (b).

(40.6 ± 9.5%). This is possibly due to the fact that the mean IgM level was higher in the "high" FATA titres group (15.6 ± 11.2 g/l) than in the "low" FATA titres group (5.7 ± 8.2 g/l) ($t = 2.84$, $p<0.01$). In fact, a positive and significant relationship was observed between the percentage of precipitated ssDNA and the serum IgM levels ($r = 0.49$, $p<0.01$) and, if one considers two groups of sera in function of the serum IgM levels, the mean percentage of precipitated DNA is higher in the "high" (10.5 to 50 g/l) IgM levels group (51 ± 8.4%) than in the "low" (0.6–10 g/l) IgM levels group (39.6 ± 8.5%) ($t = 3.74$, $p<0.001$). Similarly, the mean FATA reciprocal titre of the high IgM group (3057 ± 2043) is higher than that of the low IgM group (1012 ± 733) ($t = 3.56$, $p<0.001$). Indeed, a positive and significant relationship exists between the FATA reciprocal titres and the serum IgM levels ($r = 0.39$, $p<0.05$).

Anti-ssDNA antibodies during and after the acute phase of African trypanosomiasis

In 12 subjects, the ssDNA binding capacity was studied in sera collected at the onset of the disease (when IgM level and FATA titre were the highest) and in sera collected some months later (mean = 14.8 ± 15.7 months) when the serum IgM levels and FATA titre had returned to normal. Fig. 3 shows that the ssDNA binding capacities were lower in the 2nd sample (normal IgM) than in the first one (high IgM) for all but one patient. For these eleven subjects the mean of the differences between the percentages of ssDNA precipitated by the first and the second serum samples (18.5 ± 5.7%) is statistically significant ($t = 10.7$, $p<10^{-9}$). The only trypanosome-infected individual, whose second serum sample (collected after treatment) showed a percentage of precipitated ssDNA

(43.8%) higher than that obtained with the first serum sample (31.6%), was also the only subject whose first serum sample showed a percentage of precipitated ssDNA lower than the maximum value observed in normal subjects (34.3%) (Fig. 1).

Anti-thyroglobulin antibodies

Fifty-four sera from 21 patients were tested for anti-thyroglobulin antibodies (anti-Tg ab). Weak positive reactions at 1/10 dilution were observed in 7 sera obtained from 5 subjects. Four of these sera (drawn from two individuals) also reacted weakly (1/10 dilution) to unsensitized tanned turkey RBC. Two other sera (drawn from one individual) presented anti-Tg ab at 1/40 dilution; these sera did not react to unsensitized turkey RBC.

Discussion

The data reported in the present study show that antibodies directed against ssDNA are present in human African trypanosomiasis; conversely, no Ab directed against dsDNA could be detected. These results confirm those previously reported by Lindsley et al. (1974), the only other authors, to our knowledge, who have approached this problem in natural *T. gambiense* infection of man and are in agreement with those reported by Kobayakawa et al. (1979) dealing with the experimental *T. brucei* infection of mice. Nevertheless Lindsley and coworkers observed low (but significant) antibody responses to dsDNA in *T. rhodesiense* infections of monkeys (Lindsley et al., 1974) and rats (Lindsley et al., 1978). Three hypotheses could account for the observation of this low-level but significant anti-dsDNA activity in the course of African Trypanosomiasis. The first possibility is an increase of the non-specific precipitation of dsDNA by the sera of trypanosome infected subjects; indeed, the high IgM levels present in these sera could increase the amount of dsDNA non specifically "dragged" by the AS precipitated proteins. The second possibility involves the concept of cross-reaction between dsDNA and ssDNA; anti-ssDNA antibodies would react with determinants present in both native and denaturated DNA. This possibility seems, however, unlikely since, although the precipitation of dsDNA by most anti-dsDNA antibodies can be inhibited by ssDNA (Arana and Seligmann, 1967), the binding of radio-active denaturated DNA by anti-ssDNA antibody is competed for by unlabeled ssDNA but not at all by dsDNA (Lightfoot and Hugues, 1976). The third possibility is that this activity is actually due to anti-dsDNA antibodies that are, for an unknown reason, present at low levels during African trypanosomiasis. This possibility seems also unlikely since in our study none of these subjects presented anti-native DNA antibodies, detectable by IFAt on *Crithidia luciliae* substrate or ANA detectable by IFAT performed on rat liver sections.

Anti-DNA antibodies have also been described in two other parasitic diseases: malaria (Kreier and Dilley, 1969; Adu et al., 1982; Daniel-Ribeiro et al., 1984) and schistosomiasis (Hillyer, 1971; Jones, 1977; Danno et al., 1979; Hillyer and Rossy, 1980).

In the case of schistosomiasis, anti-DNA antibodies reacted with DNA from a variety of sources (parasite, bacterial and mammalian origin) (Hillyer, 1971) and were directed against ssDNA rather than against dsDNA (Hillyer, 1971; Jones, 1977) however, the spontaneously autoimmune strain of NZW mice infected by *S. mansoni* developed anti-dsDNA with titres higher than those exhibited by uninfected control NZW mice (Hillyer and Rossy, 1980). In addition, anti-nuclear antibodies, absorbed by calf-thymus dsDNA, have been observed in sera and kidney eluates from *S. mansoni*-infected Balb/c nude mice. Curiously, these ANA were not observed in sera or kidney eluates from infected thymus-intact Balb/c mice (Danno et al., 1979).

In murine malaria, anti-DNA antibody production has been associated with anti-RNA antibodies but no information was given about the quaternary structure of DNA (Kreier and Dilley, 1969). In human malaria, however, the anti-DNA antibodies were directed mainly against ssDNA (Adu et al., 1982; Daniel-Ribeiro et al., 1984).

This observation of anti-ssDNA autoantibodies in the course of African trypanosomiasis is particularly relevant for understanding the mechanisms of autoimmunity in this protozoan disease for two main reasons. Firstly, *Trypanosoma gambiense* has a DNA-rich kinetoplast (that has even been used to detect DNA antibodies by fluorescence methods: Thivolet et al., 1965). Release of this parasite DNA in the circulation or cross reaction with host DNA could be involved in the origin of anti-DNA antibody formation in African trypanosomiasis. Secondly, *T. gambiense* is a parasite endowed with polyclonal B-cell activating properties and it is now well documented that such substances can induce synthesis of autoantibodies such as anti-DNA antibodies in vivo (Fournié et al., 1974).

Nevertheless, we would like to draw attention to some facts that have not often been discussed. The DNA present on *T. gambiense* and other trypanosomes is bound by anti-native DNA antibody only and not by anti-denaturated DNA antibody (this property of trypanosomidae has been, in other respects, used to differentiate anti-ss from anti-dsDNA antibodies: Aarden et al., 1975). Therefore the parasite DNA could not be, at least in its original native form, directly involved in the induction of anti-ssDNA antibodies observed in the trypanosome infection.

Anti-DNA autoantibodies could also be a result of a non-specific activation of the DNA autoreactive B-lymphocytes by the parasite PBA. However, it must be kept in mind that autoreactive B-cells (ARC) specific for dsDNA and for thyroglobulin have been demonstrated to exist in similar numbers in man (Bankhurst et al., 1973; Roberts et al., 1973; Bankhurst and Williams, 1975).

These ARC could therefore constitute target cells for the PBA properties of the trypanosome and synthesize the corresponding autoantibodies if the activation of ARC were a non-specific phenomenon. In this paper, however, we have clearly shown that only antibodies directed against ssDNA but not against dsDNA nor thyroglobulin were induced.

These results suggest that the activation of ARC depends on the presence of the specific antigen and are therefore consistent with a two-signal mechanism of ARC activation; i.e. a synergistic effect of the denaturated (parasite or host) DNA and a second (non-specific) signal provided by the parasite mitogen. If this mechanism of specific ARC activation by PBA is operative, liberation (or injection) of a partially sequestered autoantigen such as thyroglobulin in the circulation of a host infected by a parasite possessing PBA properties, must be followed by the development of anti-Tg antibody. Our preliminary results concerning experimental rodent malaria seem to confirm this hypothesis.

Acknowledgments

This study was supported financially by a grant from the "Fondation pour la Recherche Médicale Française" (FRM) to CDR. Dr. C. Daniel-Ribeiro is recipient of a grant from "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq-Brazil). The authors wish to express their gratitude to Drs. F. Tron (Clinique néphrologique, Hôpital Necker, Paris) and R. Palminteri for critical review of the manuscript and Mrs. Sabine de Roquefeuil and Sheila O'Hare for their kind assistance in the preparation of this paper.

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