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CIRCULATING ANTIBODIES DIRECTED AGAINST TRYPTOPHAN-LIKE EPITOPES IN SERA OF PATIENTS WITH HUMAN AFRICAN TRYPANOSOMIASIS

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Abstract. Human African trypanosomiasis is often associated with an intense proliferation of B lymphocytes, leading to polyclonal antibody synthesis. Using a modified enzyme-linked immunosorbent assay method, we have found highly significant levels of circulating anti-conjugated tryptophan-like epitope antibodies in sera of patients with sleeping sickness. These antibodies were immunoglobulins (Ig) of the M isotype. There was no correlation between immunologic binding and the Ig levels found in sera of patients with human African trypanosomiasis. Higher antibody levels in stage II of the disease than in stage I may be related to damage to the central nervous system. The specificity of this immunologic binding was evaluated by 1) comparison with that obtained with other related conjugates and 2) serum titration. Anti-conjugated tryptophan-like epitope antibodies were not found in other neurologic diseases tested. Their involvement in this pathology remains unknown.

The pathology of human African trypanosomiasis or sleeping sickness usually comprises two stages: stage I, during which there is no evidence of central nervous system (CNS) disturbances, and stage II, defined by the presence of parasites in the CNS. Many investigators have described a dysfunction and a dysregulation of the immune system in this disease.[1] A classic immunologic feature of human African trypanosomiasis is the production of large amounts of immunoglobulins (Ig), reflecting a nonspecific polyclonal B lymphocyte activation[2] and a production of autoantibodies directed against antigens unrelated to those of the infecting parasite. Circulating autoantibodies directed against hippocampus and hypothalamus neurons have been reported in sera of infected rats.[3] Autoantibodies to the myelin structure have been described.[4–6] An increase in autoantibody titers may be due to tissue antigen release as a result of damage to the CNS by the parasite itself or inflammatory reactions, or to a large stimulation of the immune system.[5] Schultzberg and others reported that in the CNS, the trypanosomes are located in areas that have a poorly developed blood-brain barrier, i.e., the area postrema, pineal gland, and median eminence, which are periventricular organs.[7] These investigators suggested that this distribution may be related to clinical symptoms. The parasites are also found in the ependymal cells of the choroid plexus and those of the ventricular coat.[8,9] In these areas, the trypanosomes destroy the ependyma cells and the supraependyma plexus. A retrograde degeneration of serotoninergic neurons has also been noted.[10] These serotoninergic pathways are implicated in slow-wave sleep genesis. The disturbances in the serotoninergic pathways could be due to T and B autoreactive cells, such as in other autoimmune mechanisms directed against endogenous, conjugated, small-sized molecules as previously described.[11–13]

In the present study, we investigated the levels of circulating autoantibodies directed against neurotransmitters of serotoninergic pathways and various related molecules and found circulating antibodies directed against tryptophan-like epitopes of the M isotype in sera of patients with human African trypanosomiasis.

MATERIALS AND METHODS

Human sera. Sera from 76 patients with trypanosomiasis (between 3 and 78 years of age) and 22 uninfected subjects (between 3 and 68 years of age) living in Boko Songho (Bouenza focus of the Congo) were studied. Clinical examination, parasite detection, and serologic analysis were performed.

Detection of trypanosomes. This was performed in blood and lymph node fluid and, when required, in cerebrospinal fluid (CSF) by direct microscopic examination and/or using minicolumns of DEAE cellulose.[14]

Serologic analysis. Antibodies directed against trypanosomal antigens were assayed in sera using an agglutination test and an indirect immunofluorescent test.[15]

The patients were divided in two groups: group I (early blood lymphatic stage) and group II (late meningoencephalitis stage) according to the presence of parasites and number of cells (early stage $\leq$ 4 cells/mm^3 in the CSF and late stage $>$ 4 cells/mm^3). In the CSF of patients, the cells in question were lymphocytes and, in advanced late stages, plasma cells.

Sera of control groups were obtained from 10 Europeans (mean age = 28 years), 12 Africans who lived in France for more than two years (mean age = 10 years), and 12 Bolivians with Chagas' disease (mean age = 25 years).

Sera from 24 patients infected with human immunodeficiency virus-1 (HIV-1) (supplied by the Department of Infectious Diseases, Hopital Pellegrin, Bordeaux, France), classified according to the Centers for Disease Control and Prevention (CDC) (Atlanta, GA)[16] and divided into 14 HIV-positive patients and 10 patients with acquired immunodeficiency syndrome (AIDS), and sera from 12 patients with severe neurologic signs of Parkinson's disease (supplied by the Department of Neurology, Hopital Pellegrin) were also used. All of the latter patients presented with tremors, akinesia, and rigidity and were considered as being in the advanced stages of Parkinson's disease.

Conjugate synthesis. Six indoleamine molecules, tryptophan (W), 5-hydroxytrytophan (HW), 5-methoxytryptophan (MW), tryptamine (T), 5-hydroxytryptamine (HT), and

5-methoxytryptamine (MT) (all from Sigma, St. Louis, MO), and five catecholamine molecules, dopamine (DA), O-methyl dopamine, 6-hydroxydopamine, L-dopamine, and O-methyldopamine (all from Sigma) were conjugated to polypeptide carrier molecules (bovine serum albumin [BSA]; Sigma) according to published methods.[17,18] Two types of conjugates were synthesized using glutaraldehyde or anhydric glutaric acid.

Glutaraldehyde conjugates. Five milligrams of each hapten or 10 mg of BSA were dissolved in 1 ml of 1.5 M acetate buffer, pH 8. The protein solution was mixed with the hapten solution; then 200 μl of 0.5 M glutaraldehyde was added. The reaction was carried out at room temperature. A yellow color and a stable pH indicated the end of the coupling reaction. Then, 200 μl of a 10 mM sodium borohydride solution (Fluka, Buchs, Switzerland) was added to saturate the double bonds. At this point, the mixture became translucid, indicating the completion of saturation. Each solution was then dialyzed against distilled water for 24 hr at 4°C. Insoluble material was removed by centrifugation at 10,000 × *g* for 15 min. Spectral analysis of each conjugate was performed to determine the molar coupling ratio, as previously described by Geffard and others.[19] These ratios ranged between 10 and 23.

Anhydric glutaric acid conjugates. Twenty milligrams of hapten was dissolved in 200 μl of dimethylsufoxide and 800 μl of distilled water; then 170 μl of 1 M NaOH and 17 mg of anhydrous glutaric acid were mixed with hapten solution. This solution was then frozen and dried. Activation of the carboxylic group was initiated by the rapid addition of 800 μl of anhydrous dimethylformamide (Merck, Darmstadt, Germany) solution containing ethylchloroformate (Fluka) diluted 1/16. The mixture was incubated for 5 min at 4°C. A protein solution containing 20 mg of BSA in 2 ml of distilled water, and 40 μl of triethylamine (Merck) was added. The conjugates were purified by dialysis against distilled water for 24 hr at 4°C. After dialysis, absorbances at 280 and 300 nm were measured for the determination of the molar coupling ratios, which ranged between 10 and 15.

Determination of the amounts of immunoglobulins in human sera. Amounts of IgG, IgA, and IgM were evaluated using an immunonephelometric method with a BNA nephelometer (Berhing, Marburg, Germany) in sera of patients with human African trypanosomiasis (n = 40) and controls (Africans from an endemic area: n = 22, healthy European blood donors: n = 10, Africans living in France: n = 12, and subjects with Chagas' disease: n = 12).

Enzyme-linked immunosorbent assay (ELISA). A previously described ELISA method was adapted for our purposes.[19] Polystyrene well plates (Nunc, Roskilde, Denmark) were coated with 200 μl of a solution containing either an indoleamine or catecholamine conjugate or glutaraldehyde-treated BSA (BSA-G) (or anhydric glutaric acid–treated BSA [BSA-AG]) at a concentration of 10 μg/ml (optimum concentration) in 0.05 M carbonate buffer pH 9.6, for 16 hr at 4°C. After this incubation, the well plates were filled with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween), 10% glycerol, and BSA (5 g/L). The well plates were incubated for 1 hr at 37°C to saturate them and prevent the nonspecific binding of the Ig. The well plates were then rinsed twice with PBS-Tween to remove the excess nonadsorbed compounds. The well plates were then filled with 200 μl of diluted (2,000-fold) serum plus PBS-Tween containing BSA (5 g/L) and 10% glycerol. They were incubated at 37°C for 2 hr. Following this primary serum incubation, unbound antibodies were removed by two washings with PBS-Tween. Peroxidase-conjugated immunoglobulins (200 μl) were then added to the well plates. Horseradish peroxidase–conjugated goat immunoglobulins to human immunoglobulins (Diagnostic Pasteur, Marnes la Coquette, France) were diluted (20,000-fold) with PBS-Tween containing BSA (5 g/L). After this secondary antibody incubation (1 hr at 37°C), unbound antibodies were removed by two washes with PBS-Tween. The substrate solution for peroxidase assay was 0.04 g/L of *o*-phenylenediamine (Sigma) in 0.1 M citrate-0.2 M sodium phosphate, pH 5, and 20 μl of hydrogen peroxide (Sigma) added just before use in 20 ml of substrate solution. It was added to each well and after incubating for 10 min in the dark at room temperature, the reaction was stopped by the addition of 50 μl of 4 M H_2SO_4/well. The absorbance in the well plates was measured at 492 nm with a Multiskan spectrophotometer (MR 610; Dynatech Laboratories, Alexandria, VA). The specific immunologic binding of sera was obtained by substracting blank values read on well plates coated with BSA-G (or BSA-AG) from experimental absorbance values. Four assays were performed for each serum sample and good reproducibility was obtained.

Statistical methods. Results are expressed as the mean ± SEM except when otherwise stated. Comparison between group means was made using the nonparametric Mann-Whitney U test ($n < 30$). A P value less than 0.05 was considered significant.

RESULTS

Immunologic binding of sera from patients with human African trypanosomiasis using tryptophan glutaraldehyde or anhydric glutaric acid conjugates. Tryptophan was conjugated to BSA via either glutaraldehyde or anhydric glutaric acid. In the initial series of experiments, sera from African controls living in an endemic area (n = 22), patients with stage I (n = 24) sleeping sickness, and patients with stage II (n = 16) sleeping sickness were examined. For these three groups, the mean ± SEM absorbance values reflecting anti-conjugated tryptophan-like epitope antibodies were 0.713 ± 0.082, 1.227 ± 0.188, and 1.447 ± 0.233, respectively, for the glutaraldehyde conjugate (W-G-BSA) and 0.018 ± 0.006, 0.013 ± 0.005, and 0.08 ± 0.037, respectively, for the anhydric glutaric acid conjugate (W-AG-BSA). For all other indoleamine conjugates coupled to BSA via anhydric glutaric acid (T-AG-BSA, MT-AG-BSA, HT-AG-BSA, MW-AG-BSA, and HW-AG-BSA), we obtained a similar reactivity to that of W-AG-BSA. A serum reactivity was found only for indoleamine conjugates coupled to BSA via glutaraldehyde. Moreover, this immunologic binding was linked only to the IgM isotype. Thus, studies were continued on only glutaraldehyde conjugates. No binding was found after application of the anti-human IgA and IgG isotypes.

Controls experiments using BSA, glutaraldehyde (BSA-G), or anhydric glutaric acid (BSA-AG)–treated BSA were

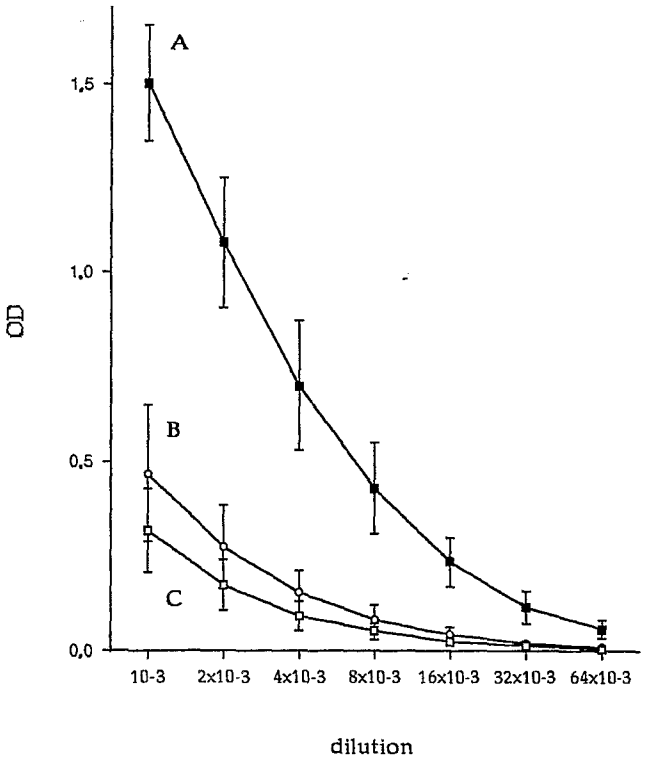

FIGURE 1. Titration curves of human sera on a glutaraldehyde tryptophan conjugate (W-G-BSA). **A,** patients with human African trypanosomiasis (n = 9). **B,** African controls living in an endemic area (n = 81). **C,** African controls living in France (n = 81). Bars show the mean ± SEM optical density (OD).

performed. The mean ± SEM absorbance values for patients and controls, respectively, were: BSA: 0.050 ± 0.009 and 0.055 ± 0.011; BSA-G: 0.114 ± 0.019 and 0.075 ± 0.018, and BSA-AG: 0.047 ± 0.008 and 0.052 ± 0.008.

Determination of immunoglobulin specificity by titration of sera from patients with human African trypanosomiasis. Serum titration was performed only on the glutaraldehyde tryptophan conjugate (W-G-BSA) with nine sera from patients with human African trypanosomiasis and sera from 16 controls (eight Africans living in France and eight Africans living in an endemic area). Two-fold serial dilutions from 1×10^{-3} to 64×10^{-3} were performed. Each sera was assayed on tryptophan conjugate (W-G-BSA) and the mean ± SEM optical density (OD) readings were then obtained for each sera group. At serum dilutions as high as 1/8,000, immunoreactivity was found only in trypanosomiasis sera (OD = 0.423 ± 0.120), whereas no binding was found with control sera (Figure 1).

Immunoglobulin levels in sera of controls and patients with human African trypanosomiasis. The amounts of IgG, IgA, and IgM in sera of patients with human African trypanosomiasis and controls were evaluated using a nephelometric procedure. Patients with human African trypanosomiasis showed increased levels of IgG (21–66.2 mg/ml) and IgM (0.96–31.2 mg/ml). This was previously described in trypanosomiasis.[20] No correlation between high antibody levels and IgM levels was found in sera of human African trypanosomiasis patients. The control group (Africans living in an endemic area) also had high levels of IgG (15.2–27.8 mg/ml) and IgM (1.57–10.7 mg/ml), but they were usually lower than in patients. The Ig increase in controls may be associated with other infections.

Study of antibody recognition with indoleamine and catecholamine conjugates. To evaluate the best immunologic binding, we assayed sera in human African trypanosomiasis with six indoleamine and five catecholamine conjugates coupled to BSA via glutaraldehyde. They were coated on well plates and sera (diluted 1/2,000) from the four control groups and the patients with human African trypanosomiasis were classified according to the stage of disease and tested. High anti-conjugated tryptophan epitope antibody levels were found in the sera of patients with human African trypanosomiasis (Figure 2). Means with 95% confidence intervals (in parentheses) were 0.702 (0.617–0.787) for African controls living in an endemic area, 1.107 (0.99–1.224) for patients with stage I disease, and 1.344 (1.166–1.522) for patients with stage II disease. These antibodies were of the M isotype. Concerning other conjugates, a low immunoreactivity was also observed for the tryptamine conjugate (T-G-BSA) but this binding was not statistically significant. Other tryptamine-like conjugates (HT [serotonin] [HT-G-SA] and MT [MT-G-BSA]) were examined but their reactivities were very low.

Low reactivity was obtained for catecholamine conjugates. No statistical difference between sera from patients with human African trypanosomiasis and sera from controls was found. For example, the mean ± SEM absorbance values for the dopamine conjugate (DA-G-BSA) were 0.185 ± 0.102, 0.145 ± 0.049, 0.170 ± 0.056, and 0.296 ± 0.114, respectively, for the African controls living in France (n = 10), the African controls living in an endemic area (n = 10), the patients with stage I disease (n = 9), and the patients with stage II disease (n = 8). Results obtained with other catecholamine conjugates were similar to those with DA-G-BSA.

Only tryptophan-like conjugates were studied further. The best response was obtained with W-G-BSA, followed by MW-G-BSA and HW-G-BSA. Analysis of results showed a highly significant difference between mean OD values of controls and those of patients for two conjugates (W-G-BSA and MW-G-BSA). The immunoreactivity of sera from patients with human African trypanosomiasis was much stronger in stage II of the disease than in stage I of the disease, but the difference between the two was not significant. High levels of anti-conjugated tryptophan-like epitope antibodies was correlated with clinical neurologic symptoms: 81.18% of the patients with major neurologic signs or 75% of the patients with stage II disease had an OD value greater than the critical value (mean + 2 SD = 1.087) calculated from reference group controls (Africans living in an endemic area) (Tables 1 and 2). Results also showed that African controls living in an endemic area had mean absorbance values higher than the other controls.

Evaluation of the specificity of this antibody binding according to different pathologic processes. To evaluate whether circulating antibodies were specific to human African trypanosomiasis, we sought the presence of such antibodies of the M isotype in sera from patients with HIV infection and Parkinson's disease. We found an increased

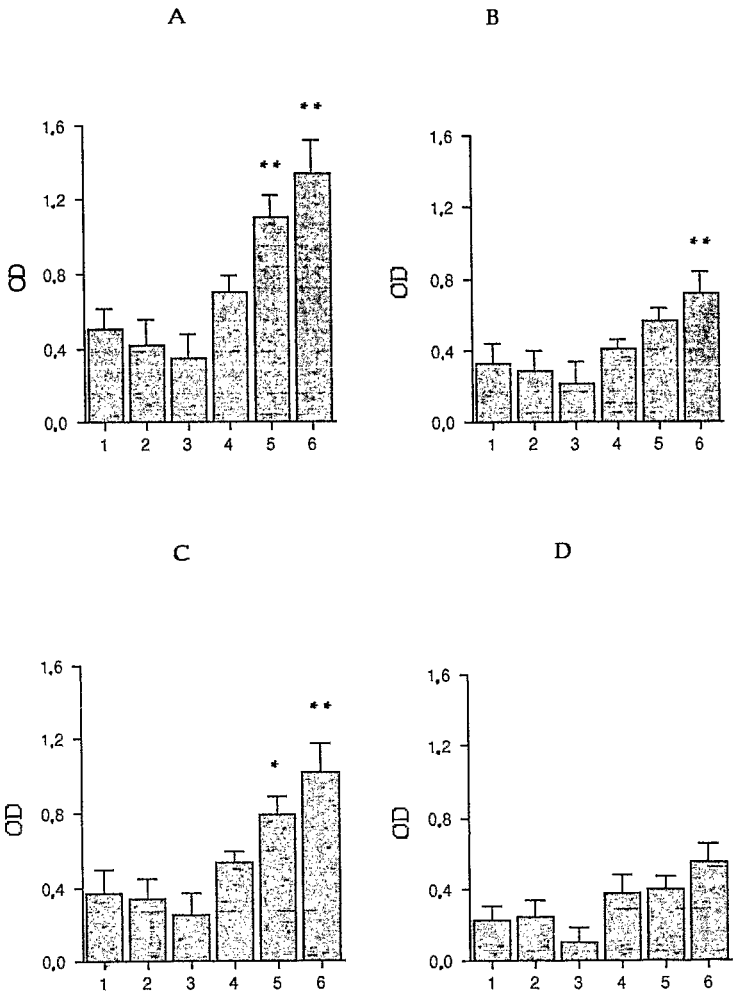

FIGURE 2. Histograms showing the optical density (OD) values reflecting levels of antibodies directed against glutaraldehyde trytophan-related molecule conjugates (**A**, W-G-BSA; **B**, MW-G-BSA, and **C** = HW-G-BSA) and a tryptamine conjugate (**D**, T-G-BSA) in sera from control groups (1 = Europeans [n = 10]; 2 = Africans living in France [n = 12]; 3 = patients with Chagas' disease [n = 12]; 4 = Africans living in an endemic area [n = 22]) and from patients with African trypanosomiasis (5 = patients with stage I disease [n = 52]; 6 = patients with stage II disease [n = 24]). Sera were diluted 1/2,000. Bars show the mean and SEM. For definition of conjugates, see Materials and Methods and Results. * $P < 0.05$ versus Africans living in an endemic area; ** $P < 0.01$ versus Africans living in an endemic area (both by the Mann-Whitney U test).

immunologic response in sera from patients with human African trypanosomiasis (0.954 ± 0.200, n = 12) with W-G-BSA. Weak immunologic binding was found in sera from patients with other diseases. No difference was detected between the two groups of HIV-infected patients. The mean ± SEM antibody levels directed against W-G-BSA, HW-G-BSA, and MW-G-BSA were 0.242 ± 0.098, 0.122 ± 0.071, and 0.169 ± 0.077, respectively, in sera from HIV-positive patients (n = 14) (Figure 3), 0.226 ± 0.083, 0.112 ± 0.048, and 0.151 ± 0.059, respectively, in sera from patients with AIDS (n = 10), and 0.220 ± 0.068, 0.126 ± 0.040, and 0.143 ± 0.043, respectively, in sera from patients with Parkinson's disease (Figure 3). These data underlined specific immunologic binding directed against tryptophan-like conjugates in human African trypanosomiasis sera.

DISCUSSION

Our results describe significantly increased anti-conjugated tryptophan-like epitope antibody levels in sera of patients with human African trypanosomiasis. This immunologic binding was related to the IgM isotype. We found reactivity of sera with the tryptophan glutaraldehyde conjugate (W-G-BSA), but not with conjugates made with glutaric anhydride acid (W-AG-BSA). This indicates the importance of the conformation of the hapten adsorbed on the well plates after conjugate synthesis. The presence of a carboxyl terminal group in tryptophan conjugates is an important element for antibody recognition because tryptamine conjugates, which have an amino terminal group, are poorly recognized (Figure 2).

Immunologic binding to glutaraldehyde-tryptophan conjugates was evaluated and a linear decrease in OD indicated the specificity of the Ig from patients with human African trypanosomiasis (Figure 1). Africans living in an endemic area had anti-conjugated tryptophan-like epitope antibody levels higher than other controls. Africans living in this endemic area might have been previously infected and then cured, trypanosome-infected but undetected, or bearers of

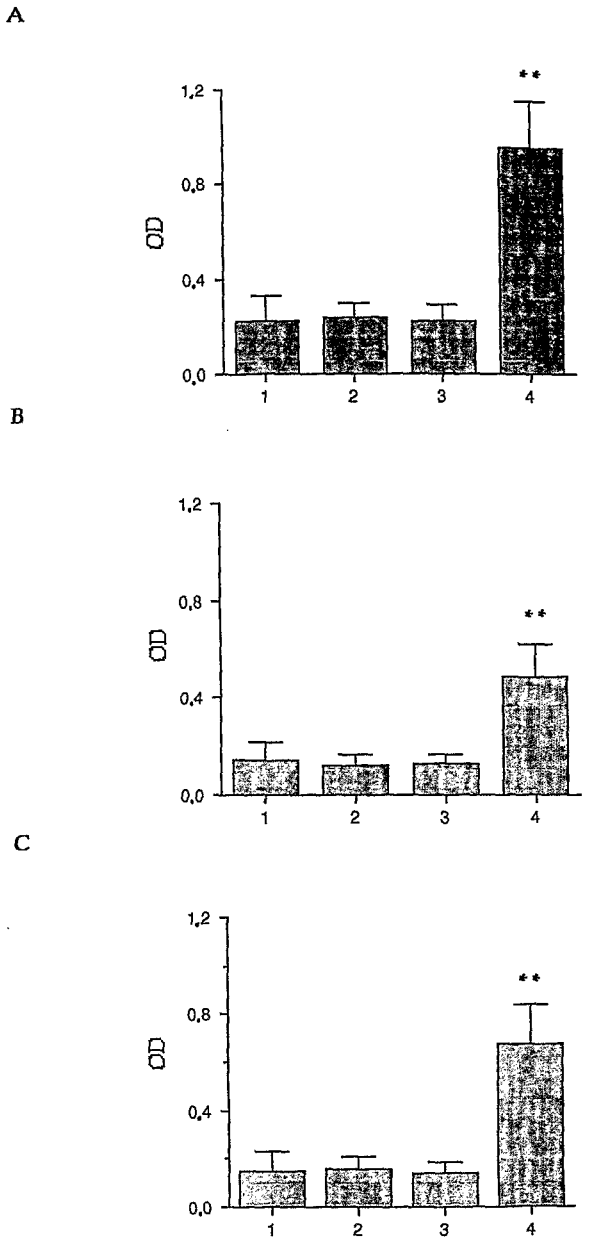

FIGURE 3. Mean and SEM antibody levels directed against glutaraldehyde tryptophan-related conjugates (**A**, W-G-BSA, **B**, HW-G-BSA, and **C**, MW-G-BSA) in sera from 1, European controls; 2, human immunodeficiency virus–positive patients; 3, patients with Parkinson's disease; and 4, patients with human African trypanosomiasis. Sera were diluted 1/2,000. Bars show the mean and SEM optical density (OD). Sera from the first three groups did not bind any tryptophan conjugates compared with sera from patients with human African trypanosomiasis. ** = 0.01, by the Mann-Whitney *U* test.

other parasites resulting in increased anti-conjugated tryptophan-like epitope antibody levels. The presence of a faint immunologic signal in European controls or Africans living in France could be a physiologic signal linked to the presence of a few autoantibodies in normal subjects or a background signal of the ELISA. We measured the Ig of serum and observed no correlation between the amount of IgM in sera from patients with human African trypanosomiasis and increased antibody levels. This agrees with the Ig specificity. Classification of sera from patients with human African trypanosomiasis according to the stage of disease and clinical symptoms showed an immunologic signal that was higher in the late stage than in the first stage and a significant correlation between high antibody levels and neurologic symptoms (Tables 1 and 2).

TABLE 1

Correlation between disease stage and anti-conjugated, tryptophan-like antibodies found in controls and in patients with trypanosomiasis detected by an enzyme-linked immunosorbent assay using tryptophan-glutaraldehyde-bovine serum albumin conjugate

Subjects	n	OD (mean ± SD)	No. > critical value* (%)†
Controls (Africans in France)	12	0.417 ± 0.212	0 (0)
Controls (Africans in endemic area)	22	0.703 ± 0.192	0 (0)
Early stage (I) of disease	52	1.107 ± 0.443	23 (44.2)
Late stage (II) of disease	24	1.344 ± 0.4242	18 (75)

* The critical value is the mean ± 2 SD optical density (OD) reading of African controls living in an endemic area.
† Percentage of patients having a mean OD reading greater than or equal to the critical value.

The increase of circulating autoantibodies in patients with human African trypanosomiasis is probably dependent on polyclonal B cell activation, but it may reflect an additional specific stimulation by autoantigens or by cross-reacting antigens.[6] High antibody titers against myelin basic protein, cerebrosides, and gangliosides have been found in murine experimental African trypanosomiasis.[21] Antibodies to myelin components (galactocerebrosides) have been found in sera of humans with African trypanosomiasis.[4] Low titers of naturally occurring anti-galactocerebroside antibodies have been reported in the sera of healthy humans, compared with higher titers observed in patients with human African trypanosomiasis.[4] These antibodies may contribute to the pathologic consequences observed. Also, experimental demyelinization has been induced by injection of anti-galactocerebroside antibodies.[22] Similar mechanisms have been proposed to explain the pathologic aspects of South American trypanosomiasis.[23] A comparison between immunologic binding in sera of patients with human African trypanosomiasis and other neurologic diseases shows a selec-

TABLE 2

Correlation between clinical symptoms and anti-conjugated tryptophan-like antibodies found in controls and in patients with trypanosomiasis detected by an enzyme-linked immunosorbent assay using tryptophan-glutaraldehyde-bovine serum albumin conjugate

Subjects	n	OD (mean ± SD)	No. ≥ critical value* (%)†
Controls (Africans in France)	12	0.417 ± 0.212	0 (0)
Controls (Africans in endemic area)	22	0.703 ± 0.192	0 (0)
Patients without clinical symptoms	35	1.031 ± 0.452	15 (42.8)
Patients with minor clinical symptoms	30	1.241 ± 0.385	19 (63.3)
Patients with major neurologic symptoms	11	1.470 ± 0.465	9 (81.8)

* The critical value is the mean ± 2 SD optical density (OD) reading of African controls living in an endemic area.
† Percentage of patients having a mean OD reading greater than or equal to the critical value.

tive increase of anti-conjugated tryptophan antibody levels only in the sera of patients with human African trypanosomiasis (Figure 3).

Indoleamine compounds are largely distributed in the CNS.[24] Serotonin (HT) is a major neurotransmitter involved in the control of numerous CNS functions, including aggressive and self-injurious behavior, regulation of sleep states and psychiatric disorders, including schizophrenia, and depression.[25] Tryptophan is the natural amino acid precursor in HT biosynthesis. It has previously been stated that HW, the first metabolite deriving directly from tryptophan, could be considered to be a putative neurotransmitter that may play a role in the regulation of the sleep/wake cycle.[26,27] Moreover, some studies have indicated the presence of a specific binding of HW to membrane receptors.[28] Thus, the multiplicity of HT receptors could be related to members of the indoleamine family, such as HW and tryptophan itself. In rat and human brains, the choroid plexus has larger amounts of HT-like receptors compared with other regions such as the pallidum or substantia nigra.[29] Supra-ependymal nerve terminals derived from serotoninergic cells have their origin in the raphe nuclei.[30] Serotonin nerve terminals are present on the walls of the ventricles and they could release HT into the CSF. During sleeping sickness, ependyma cells and the supraependymal plexus are destroyed by parasites.[10] Antibodies directed against tryptophan-like conjugates could play a role in the pathophysiology of the disease or could merely be a sign of tissue degradation devoid of effects. Their pathologic role in this disease, if any, will be clarified by further studies.

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