Human African trypanosomiasis is often associated with an intense proliferation of B lymphocytes, i.e., the area postreversal of poorly developed blood-brain barrier, i.e., the area postreversal of the infecting parasite. Circling autoantibodies directed against hippocampus and hypothalamus neurons have been described. Schultzberg and others reported that in the disease, the trypanosomes destroy the ependyma cells and the subependymal layers, 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 human African trypanosomiasis. 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 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.

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5-methoxytryptamine (MT) (all from Sigma, St. Louis, MO), and five catecholamine molecules, dopamine (DA), O-methyl dopamine, 6-hydroxydopamine, L-dopamine, and O-methylisopropyl dopamine (all from Sigma) were conjugated to polypeptide carrier molecules (bovine serum albumin [BSA]; Sigma) according to published methods. 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 acetic 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 translucent, 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. These ratios ranged between 10 and 23.

**Anhydric glutaric acid conjugates.** Twenty milligrams of hapten was dissolved in 200 µl of dimethylsulfoxide and 800 µl of distilled water: then 170 µl of 1 M NaOH and 17 mg of anhydric 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. Polystyrene well plates (Nunc, Roskilde, Denmark) were coated with 200 µl of a solution containing either an indoleamine or catecholamine conjugate or glutaraldehydetreated 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 µl 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 H2SO4 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 subtracting 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 glutaraldehyde (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
performed. The mean ± SEM absorbance values for patients and controls, respectively, were: BSA: 0.050 ± 0.009 and 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
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 formulation 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 carriers of...
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).

The immunologic signal antibodies in patients with
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.19,27 Moreover, some studies have indicated the presence of a specific binding of HW to membrane receptors.24 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.26 Supra-ependymal nerve terminals derived from serotonergic cells have their origin in the raphe nuclei.27 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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