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DETECTION AND CHARACTERIZATION OF AUTOANTIBODIES DIRECTED AGAINST NEUROFILAMENT PROTEINS IN HUMAN AFRICAN TRYPANOSOMIASIS

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Abstract. In serum and in cerebrospinal fluid (CSF) from patients with human African trypanosomiasis (HAT) with central nervous system involvement, we detected autoantibodies directed to some proteins from these tissues. The characterization of antigenic proteins by Western blotting showed that the antibodies recognized the 200-kD and 160-kD proteins of neurofilament (NF). Serum anti-NF antibodies were more frequent in HAT patients than in control subjects (86% versus 24%; $P < 10^{-9}$) and they belonged predominantly to the IgM class (anti-NF IgM = 86% versus anti-NF IgG = 4%; $P < 10^{-9}$) in the patients with stage II (central nervous system involvement) HAT. The CSF antibodies to NF were IgM in 88% (22 of 25) of the cases and IgG in 32% (8 of 25) of the cases. Epitopes shared by NF and trypanosomes were detected by indirect immunofluorescence and this was confirmed by the disappearance of anti-NF reactivity after adsorption with trypanosome antigens (*Trypanosoma brucei brucei* or *T. b. gambiense*). Anti-NF antibodies were undetectable in the CSF from stage I HAT patients.

Human African trypanosomiasis (HAT), caused by *Trypanosoma brucei gambiense* or *T. b. rhodesiense*, is transmitted by bite of the tse-tse fly (*Glossina* sp.). The disease is characterized by two consecutive stages: a lymphosanguine phase (stage I) and a meningoencephalitic phase (stage II), which is defined by the presence of more than five cells/mm^3 in the cerebrospinal fluid (CSF). In stage II, a variety of central nervous system (CNS) symptoms have been described that are related to a demyelinating meningoencephalitis, the pathogenesis of which is still undetermined and which may be due to autoimmune mechanisms.[1,2] As in Chagas' disease, the American human trypanosomiasis caused by *T. cruzi*, several autoantibodies have been described in HAT, including rheumatoid factors[3] or autoantibodies directed to red blood cells,[4] smooth muscle,[5] liver and cardiolipids[6] nucleic acids (DNA and RNA),[4,7] and intermediate filaments of the cytoskeleton in smooth muscle cells.[8] Autoantibodies directed against components of CNS myelin have also been reported. These antibodies are specific for the major glycosphingolipids of myelin, the galactocerebrosides, and they have been detected in sera from both experimentally infected animals[9] and patients from the Ivory Coast.[10] Other autoantibodies, directed against uncharacterized proteins, have been described in HAT patients[11] as well as antibodies directed against the myelin basic protein in experimentally infected animals.[7] In this study, we report the presence of autoantibodies to neurofilament (NF) proteins in sera and CSF from untreated patients with stage II HAT.

MATERIALS AND METHODS

Human sera and CSF samples. Serum samples from 50 patients with stage II HAT from Daloa, Ivory Coast and CSF from 25 of these patients were studied. Fifty healthy individuals, without clinical and biological criteria for HAT, living in the same endemic zone were analyzed in parallel as controls. The CSF from 40 stage I HAT patients were obtained from endemic areas in the Congo (Bouenza: Nkayes and Loudima Districts, Pool: Ngabe District, and Plateaux: Djambala and Gamboma Districts). The diagnosis of HAT was based on the detection of trypanosomes in blood and/or CSF and by the following serologic tests: a card agglutination test for trypanosomiasis, (CATT; Institute of Tropical Medicine, Antwerp, Belgium) and an indirect immunofluorescent antibody test, using a *T. b. gambiense* bloodstream form antigen (Institute of Tropical Medicine, Antwerp, Belgium). All samples were obtained after the informed consent of the subjects. Individual approval was obtained from all adult participants and from parents of minors by medical teams in the Ivory Coast and the Congo for diagnosis and parasitologic and immunologic HAT investigations. Only voluntary subjects were sampled, and subjects were informed that the same samples were kept for further studies to improve the knowledge of the disease. These investigations were edicted, approved, and then directed by departments depending on each concerned Health Ministry: Ministere de la Sante Publique et des Affaires Sociales, Projet de Recherches Clinique sur la Trypanosomiase (Ivory Coast) and Ministere de la Sante Publique et de la Population, Service d'Epidemiologie et des Grandes Endemies (Congo).

Reference antibodies to CNS components. Some antibodies with known specificities were used as markers of CNS proteins and glycolipids: mouse monoclonal antibodies specific for the 68-kD and 200-kD NF proteins (2F11; Dakopatts, Glostrup, Denmark) and for the 160-kD NF protein (Amersham, Les Ulis, France), mouse monoclonal antibody to the myelin-basic protein (MBP; Boehringer, Mannheim, Germany), and rabbit antiserum directed to the glial fibrillary acidic protein (GFAP; Dakopatts). We also used human IgM monoclonal antibody with autoantibody activities previously characterized as markers for either the myelin-associated glycoprotein (MAG) or the gangliosides GM1 and GD1b.[12]

Antigens. The CNS proteins were prepared from bovine white matter according to the method of Waehneldt and Mendel[13] as modified by Birling and others.[14] Briefly, bovine white matter was homogenized in 0.32 M sucrose solution containing 10 mM EDTA and 3 mM Na_2HPO_4 and centri-

fuged at 18,000 × *g* for 20 min. The pellet was washed twice with the same sucrose solution and resuspended in 0.85 M sucrose solution. The myelin fraction was isolated at the interface of the sucrose solution after centrifugation at 50,000 × *g* for 90 min, lyophilized, and extracted with by 2:1 (v/v) chloroform-methanol.[15]

Purified 200-kD NF protein from bovine brain was supplied by ICN Biomedicals (Costa Mesa, CA) and a mixture of the three purified NF proteins (68, 160, and 200 kD) from bovine spinal cord was obtained from Boehringer. Thin blood films and a pure centrifugation pellet of *T. b. brucei* strain Antwerp Trypanozoon Antigen Type (Antat) 1.1 and *T. b. gambiense* strain Lille Trypanozoon Antigen Type (Litat) 1.3 obtained from inoculated mice were kindly donated by the Central Serum Bank for Sleeping Sickness (TDR/WHO Project, Institute of Tropical Medicine, Antwerp, Belgium).

Immunochemical studies. Proteins purified from white matter were separated on 7.5%, 10%, and 12% gels by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described by Laemmli[16] and compared with molecular weight markers (Biorad SA, Ivry/Seine, France). After electrotransfer onto nitrocellulose sheets (HAHY 0.45 mm; Millipore, St. Quentin-Yvelines, France) according to the procedures of Towbin and others,[17] the blots were saturated for 2 hr at room temperature with a 3% bovine serum albumin (Sigma, St. Louis, MO) solution in phosphate-buffered saline (PBS), pH 7.2, containing 0.01% Tween 20. Human serum and CSF samples were diluted 1:100 and 1:25, respectively, with the same diluent and allowed to react as for saturation. Horseradish peroxidase–conjugated $F(ab')_2$ fragments anti-human IgG (1:2,000 dilution; Cappel, Westchester, PA) or anti-human IgM (1:1,000 dilution; Dakopatts) were then incubated for 90 min at room temperature and the final enzyme activities were revealed using 0.5 mg/ml of diaminobenzidine (Sigma) in 0.05 M Tris-HCl, pH 7.6, containing 0.01% H_2O_2. Control assays were performed with antibodies of known specificity to CNS proteins: a human anti-MAG IgM, mouse monoclonal antibodies to MBP and the 68-kD, 200-kD, and 160-kD NF proteins, and rabbit antiserum to GFAP at a 1:100 dilution. Conjugate controls were obtained by omitting human serum. The reactive human sera and CSF were also studied by a Western blotting procedure with purified 200-kD protein and a mixture of the three purified NF proteins.

Adsorption studies of human sera with anti-NF reactivity were performed with purified 200-kD NF protein (at a antigen:IgM concentration ratio of 10:4) or with trypanosome antigens (*T. b. brucei* and *T. b. gambiense*) from frozen pellets (at a antigen:IgM concentration ratio of 9:1). After adsorption, sera were tested by Western blotting with CNS proteins.

Immunocytochemical studies. The reactivity of human sera with NF proteins was determined by indirect immunofluorescence studies with the neuroblastic subclone SH-SY-5Y of the human neuroblastoma cell line SK-N-SH (kindly provided by Dr. B. Spengler, Sloan-Kettering Institute, New York, NY) cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, United Kingdom) with 10% fetal calf serum.[18] Briefly, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized with 90% ethanol for 1 min at −20°C. A 30-min saturation with 10% sheep serum (Gibco) in PBS then preceded a 1-hr incubation at room temperature with human sera diluted 1:100 in the same saturation solution. Identification of neurons was done by double-staining with monoclonal antibodies to NF proteins diluted 1:50. After further washing with PBS, the slides were stained with fluorescein-labeled rabbit IgG $F(ab')_2$ fragments to human μ chain or rhodamine-tagged rabbit antibodies to mouse immunoglobulins (Dakopatts) used at a 1:100 dilution.

Thin blood films with *T. b. brucei* (strains Antat 1.1 and Antat 1.2), *T. b. gambiense* (strain Litat 1.3), *T. b. rhodesiense* (strain Antat 25.1), and *T. evansi* (strain Rode Trypanozoon Antigen Type, Rotat 1.2) were used to search for a common reactivity between trypanosome and CNS components. After fixation with acetone, the slides were saturated as before. Antibodies to NF proteins, MAG, and gangliosides GM1 and GD1b were diluted 1:50, allowed to react for 60 min at room temperature, and revealed with fluorescein conjugates.

RESULTS

Detection and characterization of antigenic proteins. Some sera and CSF from patients with HAT reacted with the same CNS proteins. These autoantibodies were directed against two proteins with molecular masses of 200 kD and 160 kD. The 200-kD and 160-kD proteins were identified as NF proteins on the basis of their relative mobilities, which were similar to those of proteins recognized by the mouse anti-NF monoclonal antibodies (Figure 1) and by the strong reactivity of sera with the 200-kD protein (Figure 2) and the mixture of the 200-kD, 160-kD, and 68-kD purified NF proteins (Figure 3). Specificity was determined by the reactivity of these sera on neuroblastoma cells double-labeled with mouse monoclonal antibodies specific for the 68-kD and 200-kD NF proteins. Indeed, the patients' anti-NF sera stained the cytoplasm of these cells with the same pattern as the anti-NF controls (Figure 4). The abolition of this reactivity by adsorption of patients' sera with the purified 200-kD NF protein confirmed the anti-NF specificity of these antibodies, suggesting that the patients' antibodies were directed to some epitopes common to the 200-kD and 160-kD proteins. The reactivity of an irrelevant non-HAT control serum directed to another antigen (MAG) was unaffected by the adsorption procedure on the 200-kD NF protein.

Characterization of antibody isotypes. In serum, the reactivity with the 200-kD and 160-kD NF proteins was detected in 86% of the patients with HAT and in 24% of the control subjects. All reactive HAT sera contained predominantly IgM antibodies to the 160-kD and 200-kD proteins (anti-NF IgM = 86% versus anti-NF IgG = 4%; $P < 10^{-9}$) in contrast to the reactive non-HAT sera, which had IgM and IgG antibodies in the same proportions.

In CSF, the same reactivity with the 200-kD and 160-kD NF proteins was detected in 88% of the patients and this reactivity belonged to the IgM class (anti-NF IgM = 88% versus anti-NF IgG = 32%; $P < 10^{-4}$). All patients with anti-NF antibodies in the CSF had the same anti-NF anti-

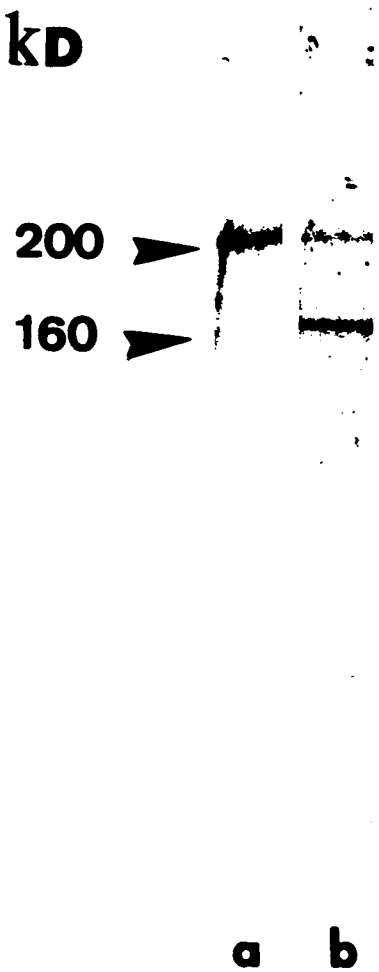

FIGURE 1. Reactivity of a patient's serum (lane b) with the 200- and 160-kD proteins from the central nervous system by Western blotting (after sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel) detected by anti-human μ chain peroxidase conjugate. Reactivity of the mouse monoclonal anti-neurofilament (NF) antibody 2F11 directed against the 200-kD NF protein (lane a) revealed by anti-mouse immunoglobulin peroxidase conjugate.

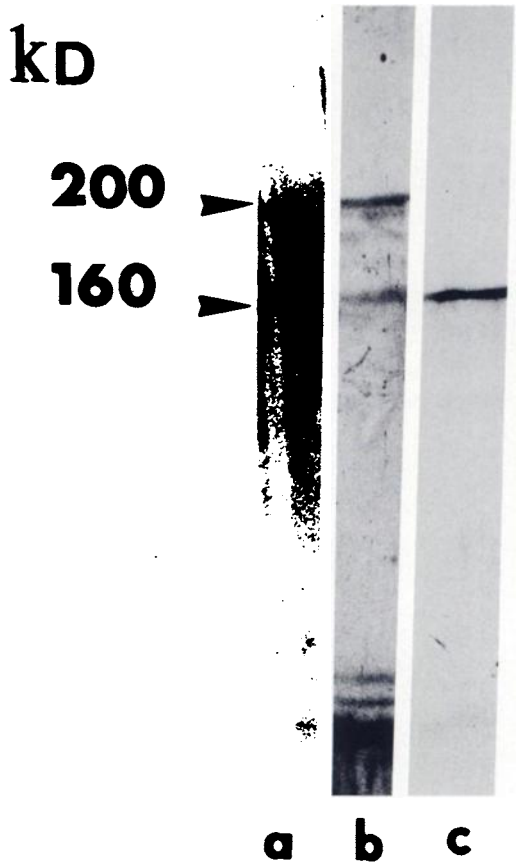

FIGURE 2. Reactivity of a patient's serum with purified 200-kD, 160-kD, and 68-kD neurofilament (NF) proteins by Western blotting (after sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 7.5% gel). Reactivity with the 200-kD and 160-kD NF proteins (lane a) detected by anti-μ chain peroxidase conjugate; reactivity of control monoclonal antibodies to 200-kD and 160-kD NF proteins (2F11, [lane b] and anti-160 kD [lane c]) detected by peroxidase-conjugated anti-mouse antiserum. The 68-kD NF protein is recognized only by monoclonal antibody 2F11 (lane b).

bodies in the serum. No reactivity with purified NF proteins was detected in CSF from stage I HAT patients.

Detection of epitopes common to NF and trypanosomes. The presence of epitopes common to trypanosome and CNS components was proved by the following results. In the indirect immunofluorescence tests on trypanosome smears performed with antibodies of known specificities directed to CNS components, the anti-160-kD monoclonal antibody showed strong reactivity with the membrane and flagellum of the trypanosomes *T. b. brucei* (Antat 1.1), *T. b. gambiense* (Litat 1.3), and *T. evansi* (Rotat 1.2) (Figure 5). Western blotting showed that the anti-160-kD and anti-200-kD NF monoclonal antibodies reacted with a trypanosome protein with a molecular weight greater than 200 kD (Figure 6). These two methods showed that no reactivity was observed with *T. b. rhodesiense* (Antat 25.1) and *T. b. brucei* (Antat 1.2). The adsorption of HAT sera with trypanosome antigens abolished the serum reactivity with the 200-kD and 160-kD NF proteins, suggesting a common epitope shared by these proteins.

DISCUSSION

In a majority of patients with HAT, we detected serum and CSF autoantibodies directed to two nervous system proteins. The identification of the specificities of these antibodies revealed that they reacted with two proteins from neuronal intermediate filaments: the 200-kD and 160-kD NF proteins, with the preponderant reactivity being against the 200-kD protein. These anti-NF antibodies belonged mainly to the IgM class.

The pathogenic significance of the anti-NF antibodies is still controversial. These antibodies have been described in sera from healthy subjects,[19-21] as seen in 24% of our control subjects, and they mainly react with NF-H (200 kD) in 27% of Caucasians, as reported by Toh and others[21] without reports of antibodies to NF in other races. Therefore, these antibodies belong to the group of natural autoantibodies directed against proteins of the intermediate filaments or to other cytoskeleton proteins, such as anti-actin, anti-tubulin, and anti-vimentin antibodies.[22,23] These natural anti-NF antibodies are mainly of the IgG class[19] or belong to the IgM

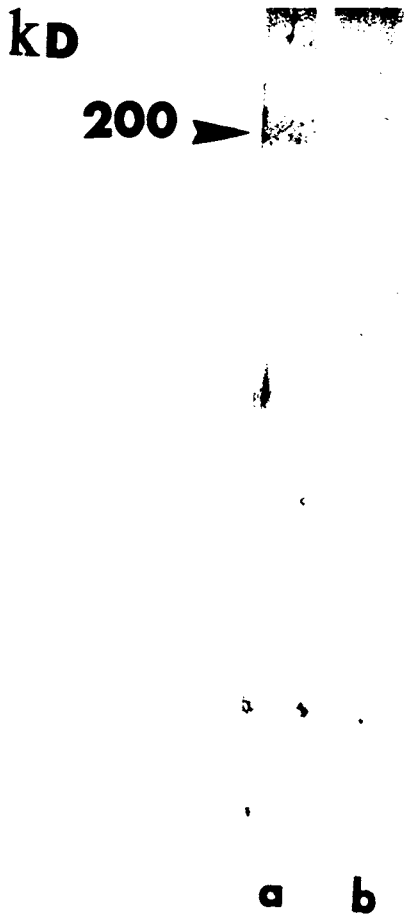

FIGURE 3. Reactivity of a patient's serum (lane a) with the purified 200-kD neurofilament protein by Western blotting (after sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel) detected by anti-μ peroxidase conjugate (control conjugate, lane b).

and IgG classes in equal proportions,[20] as in our control group.

Anti-NF antibodies have also been reported in different diseases of the nervous system. Some of them are of degenerative origin, affecting primarily the neurons (Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis).[19, 20, 24] Other diseases such as multiple sclerosis are linked to an autoimmune origin.[25] In all of these instances as in our study, the antibodies are polyclonal. In certain cases, the anti-NF activity is related to an immunoglobulin monoclonal and is associated with axon damage of the peripheral nervous system.[26–28]

The detection of anti-NF antibodies during infectious diseases, as in acute viral hepatitis,[29] could be the consequence of nonspecific stimulation of B cells producing natural autoantibodies.[30, 31] Another possibility is an immune response directed to different epitopes of the causative infecting agent with molecular mimicry to self antigens, inducing a cross-reactivity to intermediate filaments.[32, 33] In our study, the production of anti-NF autoantibodies may result from similar mechanisms. A nonspecific stimulation of B lymphocytes during HAT is suggested by the high serum immunoglobulin levels in these patients, mainly IgM (mean level = 25.8 g/L), as determined by nephelometry (Behring, Rueil-Malmaison,

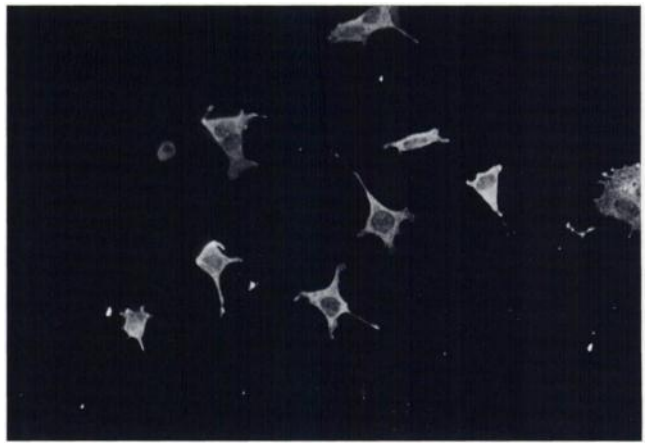

FIGURE 4. Cytoplasmic staining of neuroblastoma cells by a patient's serum containing anti-neurofilament antibodies revealed by fluorescein-conjugated anti-human μ chain (original magnification × 400).

France). The cross-reactivity between the trypanosome and NF is shown by the detection of common epitopes proven by the abolition of the anti-NF activity after adsorption of sera with trypanosome antigens and by staining of trypanosomes with anti-NF monoclonal antibodies. The pathogenicity of anti-NF antibodies in neurologic disorders occurring in HAT remains to be determined. As for the antibodies to intermediate filaments that are frequently observed during HAT[8] or the model of experimental disease in rabbits infected with *T. b. brucei*,[34] anti-NF antibodies are sometimes reactive with other intracellular components such as DNA[28] and histones[26, 35] or with the cell membrane,[28] thus potentially enabling them to cause cellular damage.[19, 28]

The reactivity with NF proteins has not previously been reported in HAT. A single study described serum autoanti-

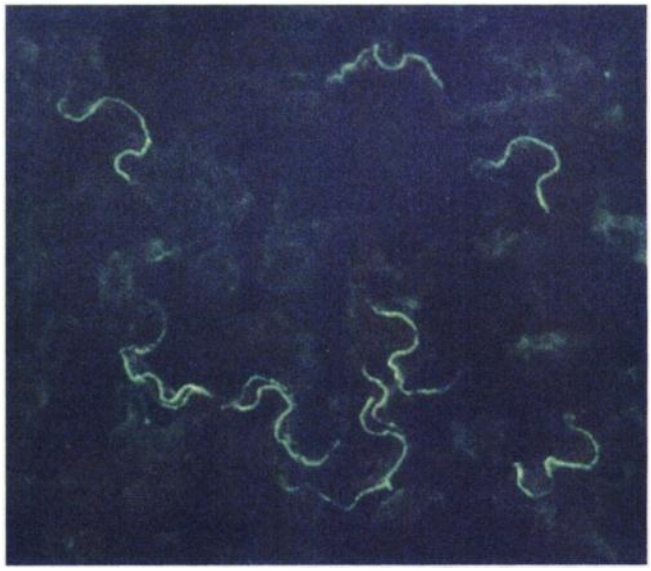

FIGURE 5. Reactivity of mouse monoclonal antibody anti-160-kD neurofilament protein with flagellum and undulating membrane of trypanosomes by indirect immunofluorescence with fluorescein-conjugated anti-mouse immunoglobulin antibodies (original magnification × 400).

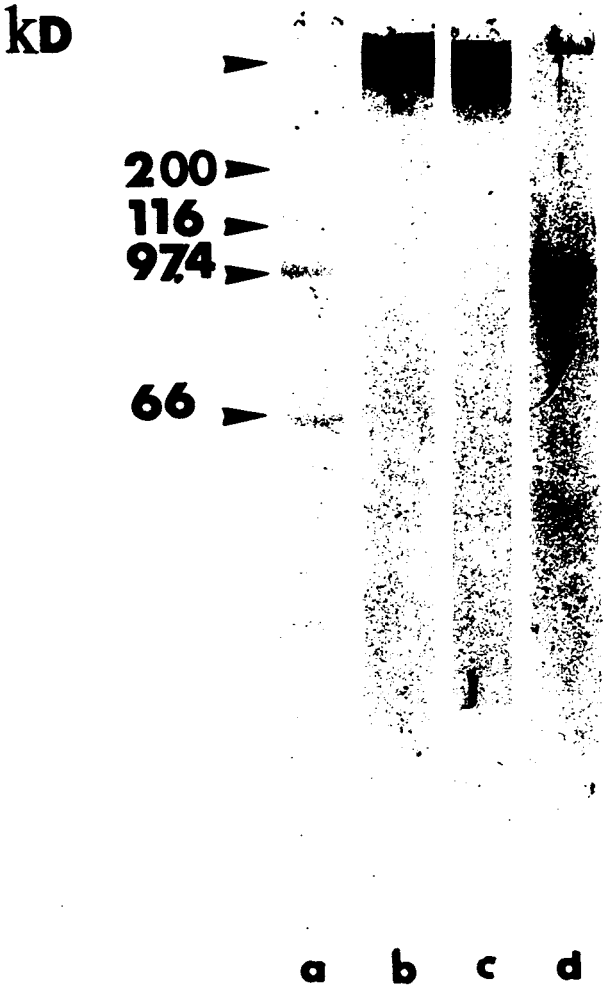

FIGURE 6. Reactivity of mouse monoclonal antibodies anti-200 kD (lane b) and anti-160 kD (lane c) neurofilament proteins with trypanosome proteins from *Trypanosoma brucei gambiense* strain Litat 1.3 by Western blotting (after sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 10% gel) and control anti-mouse immunoglobulin peroxidase conjugate (lane d). Lane a = molecular weight markers.

bodies directed to CNS proteins in 23.1% of the sera of patients with stage I HAT and in 71.4% of those with stage II HAT.[11] These investigators described a preponderant reactivity to a 25-kD protein of antibodies belonging to both the IgG and IgM classes. The detection of antibodies recognizing epitopes expressed by the proteins of neuronal cytoskeleton and by trypanosomes suggests that the microtubular structure of the undulating membrane and flagellum of the trypanosome[36, 37] might contain peptide sequences common to NF.

The finding of anti-NF IgM autoantibodies in the CSF of 88% of the patients with stage II HAT led us to search for these autoantibodies in the CSF from 40 stage I patients from the Congo. No anti-NF IgM antibodies were detected in samples from these patients by Western blotting. These data argue for the possible participation of these autoantibodies in the neurologic involvement associated with stage II of this disease. However, the pathogenetic mechanisms of these antibodies to NF in HAT remain to be determined.

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