Cysticercus Antigens in Cerebrospinal Fluid Samples from Patients with Neurocysticercosis

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Antigens were detected in cerebrospinal fluid (CSF) samples from patients with neurocysticercosis (NC) by enzyme-linked immunosorbent assay (ELISA) using polyclonal sera of rabbit anti-Taenia solium cysticerci (anti-Tso) and anti-Taenia crassiceps cysticerci vesicular fluid (anti-Tcra or anti-Tcra<30 kDa). A group of NC patients (n = 174) were studied (NC), including 40 patients in different phases of the disease. ELISAs carried out with the anti-Tso, anti-Tcra, and anti-Tcra<30 kDa showed sensitivities of 81.2, 90, and 95.8% and specificities of 82, 98, and 100%, respectively. The 14- and 18-kDa low-molecular-weight peptides were only detected in CSF samples from patients with NC by immunoblotting with anti-Tso and anti-Tcra sera. Because of the importance of the diagnosis and prognosis of cysticercosis, the detection of antigens may contribute as an additional marker to the study and clarification of the parasite-host relationship.

Cysticercosis, caused by the larval form of *Taenia solium* in tissues and organs of pigs and, accidentally, humans, represents an important health problem with socioeconomic repercussions. About 50 million people in the world are estimated to have cysticercosis, and about 50 thousand die of the disease every year (3). It is considered an endemic disease in underprivileged regions of Latin America, Asia, Africa, China, and Indonesia and is of concern to authorities in developing countries (23, 31, 34).

The most severe form of the human infection, i.e., neurocysticercosis (NC), results from the presence of cysticerci in the central nervous system and shows severe symptoms such as epilepsy, psychic and demential signs and symptoms, and increased intracranial pressure, the last condition being responsible for the high lethality of the disease (21). Imaging examinations such as computed tomography and magnetic resonance imaging are the most effective methods by which to detect cysts in all phases of the disease, as well as an inflammatory response, but these techniques are very expensive and inaccessible to most of the affected population (8). Rapid and simple tests are therefore necessary, including those employed for epidemiologic studies (11, 18, 20, 25). Immunological methods have been used for the detection of anti-cysticercus antibodies in cerebrospinal fluid (CSF) and serum. Several investigators have demonstrated the use of antigen preparations especially purified from glycoprotein fractions for the detection of anti-*Taenia solium* antibodies (13, 16, 30). Our group has studied the use of *Taenia crassiceps* antigens as an alternative source and their antibodies (13, 16, 30). Our group has studied the use of purified from glycoprotein fractions for the detection of anti-cysticercus antibodies.

Parasites and antigens. Vesicular fluid antigen from the larval form of *T. crassiceps* (VF-Tcra) strain ORF (14) and *T. solium* total saline antigen (T-Tso) were obtained as follows. Intact parasites of *T. crassiceps* were ruptured and centrifuged at 15,000 × g for 60 min at 4°C, and the supernatants were sonicated at 20 kHz and 1 mA for four periods of 60 s each in an ice bath. The supernatant obtained after further centrifugation represented VF-Tcra. After lyophilization, intact *T. solium* cysticerci were reconstituted with saline solution (1 ml/100 mg of powder) and homogenized in an ice bath for 5 min and the supernatants were treated as described before. The supernatant obtained after further centrifugation represented T-Tso. Phenylmethylsulfonyl fluoride (Sigma Chemical Company, St. Louis, Mo.) was added to each antigen extract at a final concentration of 4 × 10⁻¹ mM.

Isolation and fractionation of immunosera. A group of six rabbits were immunized with the T-Tso, VF-Tcra, and Tcra<30 kDa antigens. The Tcra<30 kDa antigen was prepared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, with only the strip representing a molecular mass of less than 30 kDa being cut out of the gel. Each rabbit was immunized with 100 μg of antigen protein in Freund’s complete adjuvant in a final volume of 1 ml. After 15 days, another dose in Freund’s incomplete adjuvant was applied. Blood was collected on days 30 and 45. The immune sera were fractionated to obtain the immunoglobulin G (IgG) fraction as described by McKinney and Parkinson (22). The immune sera were diluted with 4 volumes of 60 mM acetate buffer, pH 4.0, and the
pH was adjusted to 4.5. Caprylic acid (25 μl/ml) was slowly added dropwise with thorough mixing, and the solution was centrifuged at 10,000 × g for 30 min. The supernatant was filtered and mixed with 1/10 volume of 10×-concentrated phosphate-buffered saline (PBS); and the pH was adjusted to 7.4. The supernatant was cooled to 4°C and fractionated with ammonium sulfate (0.277 g/ml), and the sample was stirred for 30 min before the precipitated IgG was collected by centrifugation at 5,000 × g for 15 min. The IgG pellet was resuspended in PBS and dialyzed against PBS.

Samples. The protocol was approved by the Ethics Committee for the Analysis of Research Projects of the Clinical Director's Office of the Hospital (approval no. 072/97). All of the patients in the NC group had a diagnosis of NC on the basis of the criteria of the General NC Investigation Protocol of the Hospital of the Faculty of Medicine, University of São Paulo. A total of 104 CSF samples from patients with a diagnosis of NC were analyzed. For 40 patients, it was possible to obtain results of imaging exams, with 27 of them being classified as the active form (cysts associated with an inflammatory process) and 13 being classified as the inactive form (nodular calcifications). All of these 40 patients had been clinically followed up for periods of time ranging from 2 to 10 years. Seventy CSF samples were obtained from patients in the control group with a negative clinical laboratory diagnosis of NC.

ELISA. Plates with 96 wells (Nunc) were sensitzed with 50 μl of CSF plus 50 μl of 0.02 M carbonate-bicarbonate buffer, pH 9.6, for 18 h in a humidified chamber at 4°C. The plates were blocked with 5% skim milk (Molico skim milk; Nestlé, Aracatuba, São Paulo, Brazil) in 0.01 M PBS (0.0075 M Na2HPO4, 0.025 M NaH2PO4, 0.14 M NaCl, pH 7.2) containing 0.05% Tween 20 (Merck, Schuchardt, Munich, Germany) (PBS-T). The ideal immune serum and conjugate concentrations were obtained by titration. We diluted control (nonimmune rabbit) serum to 1:100, anti-Tcra serum to 1:50, and anti-Tso serum to 1:100 and added peroxidase-labeled rabbit anti-IgG (Sigma Chemical Co.). The enzymatic reaction was developed with the chromogenic substrate tetramethylbenzidine and hydrogen peroxide (Bio-Rad Laboratories, Inc., Hercules, Calif.) for 20 min in the dark and blocked with 4 N sulfuric acid. Labeling intensity was quantified with a plate reader at 450 nm (Diagnostics Pasteur, Strasburg-Schiltigheim, France). The absorbance (optical density [OD]) obtained for each test was subtracted from the control (nonimmune rabbit) reading. All incubations were carried out at 37°C for 1 h, except for the blocking step, which was carried out for 2 h. Between the sample, conjugate, and substrate incubation steps, the plates were washed in an automatic washer with four cycles of saline solution containing 0.05% Tween. All plates contained a control with T-Tso and VF-Tcra

FIG. 1. ELISA results, expressed as ODs, for the detection of antigens in 104 CSF samples from the NC group and 70 from the control group assayed with anti-T. solium cysticercus (Tso), anti-T. crassiceps cysticercus (Tcra) and anti-T. crassiceps <30 kDa (Tcra <30) sera. The cutoff points for the reactions are shown as horizontal lines, and the numbers of samples assayed are shown at the bottom.
The sera used in the present study for the detection of antigens in CSF samples from patients with NC were found to be efficient. The hyperimmune sera obtained from the heterologous *T. crassiceps* antigen showed the highest sensitivity and specificity in ELISA, reaching sensitivities of 81.2, 90, and 95.8% and specificities of 82, 98, and 100%, respectively. No difference between anti-Tso and anti-Tcra sera was observed in the 40-sample group with image diagnosis. Antigens were detected in 100% of the samples from patients with the active form by using the two sera; antigens were detected in 76.9% of the inactive-form samples assayed with anti-Tso serum and in 92.3% of the samples assayed with anti-Tcra serum.

Two reactive samples from the NC group and two from the control group were assayed by immunoblotting for antigen characterization. The 14- and 18-kDa peptides were only identified in samples from the NC group, while the 34-kDa protein was considered nonspecific since it was also identified in control samples (Fig. 2).

The sera used in the present study for the detection of antigens in CSF samples from patients with NC were found to be efficient. The hyperimmune sera obtained from the heterologous *T. crassiceps* antigen showed the highest sensitivity and specificity in ELISA, reaching sensitivities of 81.2, 90, and 95.8% and specificities of 82, 98, and 100% for the anti-Tso, anti-Tcra, and anti-Tcra <30 kDa sera, respectively, with 91.5% concordance. The cutoff points (T-Tso, 0.68; VF-Tcra, 0.48; Tcra <30 kDa, 0.81) were chosen in order to obtain higher specificity than sensitivity. This high background may be due to nonspecific binding of the conjugate to the microplates (Maxisorp) or to minimal cross-reactivity with adsorbed human IgG from the samples.

Other authors, using anti- *T. saginata* monoclonal antibodies, detected antigens in CSF samples (4, 7) and in sera from humans and infected cattle (1, 9).

Anti-Tso sera have been used for the detection of antigens in CSF samples from patients with NC. Tellez-Giron et al. (29) using dot-ELISA and ELISA, showed that 59 and 77%, respectively, of 17 CSF samples from patients with NC contained antigens. Velasco-Castrejon et al. (33) detected *T. solium* antigens in 88% of 215 CSF samples from patients with NC by agglutination of latex particles adsorbed to anti-vesicular fluid, anti-excretion-secretion, and anti-total *T. solium* cysticercus extract immunoglobulins.

In the present study, the 34-kDa peptide was considered to be nonspecific since it was also identified in control samples (Fig. 2). Low-molecular-mass peptides (<20 kDa) have been identified by antibodies in samples from patients with cysticercosis (15, 17, 26, 27, 30). Our group has recently reported that the 14- and 18-kDa peptides are responsible for the cross-reactivity between the *T. solium* and *T. crassiceps* species (10) and are specific for antibody detection in serum and CSF samples from patients with cysticercosis (2). In the present study, these peptides were strongly recognized in the two CSF samples using anti-Tso and anti-Tcra sera in immunoblots, suggesting that they may interact more intensely with the host, possibly representing excretion and secretion products released into CSF during the different phases of the parasitic evolution of NC (active and inactive forms).

In contrast to the present results, other investigators detected high-molecular-mass peptides by using anti-Tso serum. Tellez-Giron et al. (29) characterized a circulating antigen of 66 kDa in CSF samples. Estrada et al. (12) identified two antigens of 190 and 230 kDa in 14 of 18 CSF samples from 82, 98, and 100% for the anti-Tso, anti-Tcra, and anti-Tcra <30 kDa sera, respectively, with 91.5% concordance. The cutoff points (T-Tso, 0.68; VF-Tcra, 0.48; Tcra <30 kDa, 0.81) were chosen in order to obtain higher specificity than sensitivity. This high background may be due to nonspecific binding of the conjugate to the microplates (Maxisorp) or to minimal cross-reactivity with adsorbed human IgG from the samples.

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patients with suspected NC and in the cysticercus vesicular fluid. Choromanski et al. (5) identified two antigens of 110 and >400 kDa in CSF samples by high-performance liquid chromatography.

Some authors have suggested that the detection of antibodies against low-molecular-mass peptides may be associated with the developmental phase of the parasite (6, 24, 28, 35), whereas Bueno et al. (2) did not find an association between antibody detection and the phase of the disease. The detection of antigens in patients in different phases of the disease, as analyzed in the present study, revealed practically the same reactivity with anti-Tso and anti-Tcr sera. It is important to note that the difficulty in detecting larval antigens in CSF may be related to their low concentration, antigen degradation, or the release of a still unidentified antigen. Among the 40 samples from patients with imaging results, antigens were identified in 100% of the cases of the active form with anti-Tso and anti-Tcr sera, whereas 3 (23%) of the 13 cases of the inactive form showed a negative result with anti-Tso serum and one (8%) of these samples was also negative with anti-Tcr serum. Anti-Tcr serum was more sensitive for diagnostic purposes even during the calcification phase (92%), and anti-Tso serum could be used to distinguish the disease phase in a more adequate manner. It should be pointed out that the patients under study had been followed up for periods of 2 to 10 years and those in the calcification phase were in the initial part of this process, which may last several years. Verification of the test with a larger number of samples is required to determine whether there is a correlation with the developmental phase of the parasite or with the host’s immune inflammatory process.

The sera used in the present study proved to be efficient for antigen identification in CSF samples from patients with NC, suggesting that antigen identification may contribute as an additional marker to the study and understanding of the disease, in addition to being of help in the diagnosis and prognosis of cystercerosis.

In future studies, analysis by immunoblotting using sensitive systems for protein detection such as enhanced chemiluminescence (Amersham Pharmacia Biotech) and monoclonal antibodies for various antigenic epitopes may define the peptides present during different phases of the infection.

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