Evaluation of an Immunoenzymatic Assay Detecting Specific Anti-Toxocara Immunoglobulin E for Diagnosis and Posttreatment Follow-Up of Human Toxocariasis

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Toxocariasis is a parasitic zoonosis caused by infestation of humans with larvae of Toxocara canis, the common roundworm in dogs, and possibly, larvae of T. cati, a common ascarid species of cats (16, 22). Two syndromes have been identified: visceral larva migrans (3) and ocular larva migrans (21). Results of recent studies suggest that the disease frequently assumes the features of a syndrome that comprises chronic weakness, abdominal pain, various signs of allergy, and hypereosinophilia (11, 18, 30). Moreover, toxocariasis has been found to be a cofactor for epilepsy (1) and has been proposed as a possible etiology in various neurologic syndromes (25).

These data have been obtained by sensitive and specific serodiagnostic methods by using excretory-secretory antigens from T. canis larvae (TES-Ag) (6). These methods are the TES-Ag enzyme-linked immunosorbent assay (TES ELISA) (7, 14) and, more recently, the Western blot (WB; immunoblot) procedure (20), both of which detect immunoglobulin G (IgG) specific for TES-Ag. However, clinical signs of allergy and increased levels of total IgE in many cases of toxocariasis (11, 13, 18) indicated that IgE specific for TES-Ag (sIgE) would be present. Brunello et al. (5) and Desowitz et al. (8) detected IgE antibodies for larval extracts of T. canis in patients with toxocariasis and asthmatic children. In 1986, Genchi et al. (10) used a radioimmunoassay with TES-Ag for the immunodiagnosis of toxocariasis and showed that this assay is valuable for the diagnosis of ocular larva migrans (9). In 1986, Oliver et al. (23) demonstrated that sIgE is detectable by ELISA.

In most countries, strict legal and technical regulations are applied to radioimmunoassays that use isotopically labeled reagents. Thus, we perfected for the first time an ELISA for the detection of sIgE (sIgE ELISA); we then assessed the specificity of this test in sera from patients presenting with various allergic or helminthic diseases or containing immune anti-blood group antibodies. Then, we studied the value of sIgE ELISA for the serodiagnosis of toxocariasis in sera from patients suspected of having the disease. The serodiagnosis of this zoonosis was based on the results of a WB assay that detected IgG for TES-Ag. WB was chosen since it is well correlated with TES ELISA; moreover, it seemed more specific. Banding patterns that included low-molecular-weight bands appeared to be specific for toxocaral infestations; high-molecular-weight bands were found to be present at significant levels only when sera from patients with various helminthic diseases were tested, 33% of which yielded positive results by TES ELISA (20). Finally, the effectiveness of sIgE ELISA for use in follow-up assessments of treated patients was evaluated.

MATERIALS AND METHODS

TES-Ag. Cultivation of T. canis larvae was carried out by the prototype method of de Savigny (6) that was modified by Bowman et al. (4). In culture vials, the larval density was 1,000 larvae ml−1, and the RPMI 1640 medium (Intermed, Vénissieux, France) in the vials, supplemented with 1% glutamine, was renewed every week. The supernatants were filtered on Whatman no. 1 filter paper and were frozen at −70°C. After thawing, the batches were pooled, dialyzed for 48 h in distilled water (Visking dialysing tube C 75; Poly Labo, Strasbourg, France), and then freeze-dried in 5-ml vials. Before each pool was used, the protein titer was measured by the method of Lowry et al. (17).

Positive and negative reference samples. A positive refer-

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ence serum pool was made from the sera of 30 patients presenting with toxocariasis that was positively serodiagnosed by WB, namely, a typical seven-band pattern (20). A negative reference serum pool was made from the sera of 30 children (age, 3 to 6 months) referred to the Laboratory of Parasitology by the Department of Paediatrics for toxoplasmosis serology. Titers of total IgE in the positive and negative reference pools were 2,760 and 3.1 kIU liter⁻¹, respectively, as determined by radioimmunoassay in the Department of Nuclear Medicine, Centre Hospitalier Universitaire Purpan. Evaluation of the reproducibility of the sIgE ELISA was done by testing positive and negative reference pools two times a week for 19 weeks.

**Human sera from patients with allergic diseases (group 1).** Sera were obtained from 41 patients attending the Department of Dermatology and Allergy for various symptoms of allergy (asthma [n = 15], eczema [n = 16], or chronic urticaria [n = 10]). Group 1 patients exhibited sensitization to various allergens (antibiotics, house dust mites, pollens, and foods) as shown by radioallergosorbent tests, patch tests, or prick tests. Only seven patients had atopic eczema. The mean total IgE value was 972.4 kIU liter⁻¹ (95% confidence interval [CI], 555.1, 1,499.6; range, 10 to 11,000 kIU liter⁻¹).

**Human sera with immune anti-A and/or anti-B blood group or heterophilic antibodies (group 2).** Sera were obtained from 12 patients with immune anti-A hemagglutinins, 3 patients with both anti-A and anti-B immune antibodies, and 5 patients with heterophilic anti-sheep erythrocyte antibodies.

**Human sera from patients with various helminthic diseases (group 3).** We collected in the Laboratory of Parasitology 175 serum samples from patients with anisakiasis (n = 2); cystic hydatidosis (n = 7); fascioliasis (n = 5); various filariases (n = 22); a history of living in an African country, hypereosinophilia, and a negative parasitological checkup but a positive serodiagnosis for filariasis by fluoroimmunoassay (n = 22); various schistosomiasis with a positive serodiagnosis and/or direct evidence of parasite eggs (n = 24); strongyloidiasis with a positive stool examination (n = 28); hypereosinophilia with a negative parasitological checkup except positive serodiagnosis for strongyloidiasis (n = 9); and positive stool examination for *Ascaris lumbricoides* (n = 7), hookworms (n = 6), pinworms (n = 22), tapeworms (n = 9), and whipworms (n = 12).

With these sera, WB with TES-Ag was negative or displayed the high-molecular-weight banding pattern only.

**Human sera from patients presenting with a clinical and/or a biological syndrome evocative of toxocariasis (group 4).** Sera were collected from 150 patients attending the Consultation Office in the Laboratory of Parasitology. None of the group 4 patients had ever lived outside of France; only 3 patients had traveled overseas as tourists. For all patients repeated stool examinations, including examinations by Baer man's method, were negative for intestinal helmintism, as were serological checkups for the autochthonal parasitic diseases cystic hydatidosis, fascioliasis, and strongyloidiasis. A serodiagnostic test for trichinosis is no longer included in the panel of tests for autochthonal parasitic diseases. From 1975 to 1977, serodiagnostic tests for trichinosis were systematically carried out on over 12,000 serum samples referred to the Laboratory of Parasitology, but only two cases of covert trichinosis were detected. For the three patients who had traveled overseas, parasitologic examinations of blood, skin, and urine were found to be negative, and serodiagnostic tests for tropical diseases (i.e., amoebiasis, filariasis, malaria, and schistosomiasis) were also negative. In 138 patients, serodiagnostic tests for toxocariasis by WB exhibited a typical seven-band pattern that included low-molecular-weight bands. For 12 patients, results of WB remained negative.

The date of onset of toxocariasis was estimated. On average, the duration of the disease before consultation at the Laboratory of Parasitology was 4.8 months (95% CI, 4.1, 5.6). The main clinical features were chronic weakness (76%), abdominal pain (36.5%), cough (26.5%), and various signs of allergy, the most frequent of which was pruritus (40%). The clinical level of illness was quantified by using a clinical score system performed in the Laboratory of Parasitology (mean, 7; 95% CI, 6.2, 8.2). The mean eosinophil count was 784 cells μl⁻¹ (95% CI, 676, 901), and the mean level of total IgE was 510 kIU liter⁻¹ (95% CI, 399, 653).

**Human sera from patients included in a therapeutic trial (group 5).** Sera from 60 patients from group 4 were obtained for inclusion in a therapeutic trial. Twenty-eight patients were treated with diethylcarbamazine (DEC), and 32 patients were not treated with DEC. These two batches of sera were similar since, before treatment, no significant difference was found (Mann and Whitney's test) between the values of duration of disease before consultation, clinical score, eosinophil count, WB results, and total and specific IgE levels. According to epidemiologic findings (11), the following advice was given to patients so that they could avoid possible reinfection: deworming of any pet three times a year, enclosing kitchen gardens, and careful hand-washing before meals. These patients were examined again for a control checkup from 2 to 3 months after the first consultation.

**WB procedure.** The WB procedure used in this study has been described elsewhere (20). Briefly, electrophoresis of TES-Ag was carried out in a gel containing 10% polyacrylamide and 7.5 μg of TES-Ag per lane. Transfer (semi-dry type) lasted for 45 min under 0.2 to 0.4 V cm⁻¹. For immunodetection, sera were diluted at 1:100. For that procedure, we used Auroprobe BL Plus and Intense BL kits (Amersham, Paris, France), in which anti-human IgG was labeled with gold and the detection of immune complexes was enhanced by a silver coprecipitation.

**sIgE ELISA procedure.** Ninety-six-well microtitration polystyrene plates (Immunoplate, Poly-Labo Block; Nunc, Strasbourg, France) were loaded with a TES-Ag solution (50 μl per well) containing 2.5 μg of protein ml⁻¹ in a 0.1 M carbonate-bicarbonate buffer (pH 9.6). The plates were maintained for 18 h at 25°C in a moist chamber. Then, the antigenic solution was discarded and the plates were washed three times with phosphate-buffered saline (PBS)-TWEEN (0.05% Tween 20; pH 7.2) buffer; they were dried and finally stored at −70°C. All test reagents, including anti-human IgE conjugate labeled with β-galactosidase, were part of the Phadezym Rast kit (Pharmacia, Bois d'Arcy, France). Sera from patients and the negative reference pool were tested undiluted; positive reference pool sera were tested undiluted and at 1:10, 1:100, and 1:1,000 dilutions. All diluted and undiluted sera were tested in duplicate. Sera (50 μl per well) were incubated at 25°C for 3 h. The plates were washed three times with PBS-TWEEN (0.05% Tween 20; pH 7.2) buffer. Fifty microliters of the anti-IgE conjugate solution was added to each well, and the plates were maintained at 25°C for 16 h in a moist chamber. Then, they were washed three times with PBS-TWEEN buffer. A total of 150 μl of the substrate (o-nitrophenyl-β-D-galactopyranoside solution) was added to each well, and the plates were incubated at 37°C for 2 h. Finally, the reaction was stopped by the addition of 50 μl of the blocking solution to each well. The
TABLE 1. Assessment of the cross-reactivity of sIgE ELISA

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients whose sera were infected with the following titers (TU liter⁻¹):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥1</td>
</tr>
<tr>
<td>1 (allergic patients)</td>
<td>41</td>
</tr>
<tr>
<td>2 (immune isohemagglutinin)</td>
<td>20</td>
</tr>
<tr>
<td>3 (helminthiasis)</td>
<td>175</td>
</tr>
<tr>
<td>Anisakiasis*</td>
<td>2</td>
</tr>
<tr>
<td>Cystic hydatidosis*</td>
<td>7</td>
</tr>
<tr>
<td>Fascioliasis*</td>
<td>5</td>
</tr>
<tr>
<td>Filariasis caused by:</td>
<td></td>
</tr>
<tr>
<td>Bancroftiasis</td>
<td>22</td>
</tr>
<tr>
<td>Loiasis</td>
<td>3</td>
</tr>
<tr>
<td>Mansonella perstans</td>
<td>4</td>
</tr>
<tr>
<td>Mansonella streptocerca</td>
<td>6</td>
</tr>
<tr>
<td>Onchocerciasis</td>
<td>1</td>
</tr>
<tr>
<td>Occult filariasis*</td>
<td>22</td>
</tr>
<tr>
<td>Schistosomiasis*</td>
<td>24</td>
</tr>
<tr>
<td>Strongyloidiasis*</td>
<td>28</td>
</tr>
<tr>
<td>Strongyloidiasis*</td>
<td>9</td>
</tr>
<tr>
<td>Intestinal helminthiasia*</td>
<td>56</td>
</tr>
<tr>
<td>Ascaridiasis</td>
<td>7</td>
</tr>
<tr>
<td>Hookworms</td>
<td>6</td>
</tr>
<tr>
<td>Pinworms</td>
<td>22</td>
</tr>
<tr>
<td>Tapeworms</td>
<td>9</td>
</tr>
<tr>
<td>Whipworms</td>
<td>12</td>
</tr>
</tbody>
</table>

Total 236 109 68 41 24 10

* Diagnosis by direct evidence of parasite.
* Diagnosis by serology.

plates were read at 420 nm with a Multiskan photometer (Flow Laboratories, Puteaux, France).

The following steps were done with software written in the Laboratory of Parasitology. The software was compiled in GW-Basic. This software read the optical densities (ODs) from every pair of wells and calculated the means. When a difference of over 10% was found between the two ODs corresponding to the same pair of serum samples, the pair of serum samples was discarded and tested again. The negative pool was read at first and its average OD at 420 nm (OD420) was subtracted from the values of other sera. Then, an undiluted positive reference pool of sera and 1:10, 1:100, and 1:1,000 dilutions of serum were read, and a positive reference curve was constructed. Results for patient sera were expressed in units. According to Voller et al. (31), this method yields the second best result when various systems that give ratios for positive versus negative serum samples were tested. In Pharmacia's system of units, the positive reference serum was heterologous and contained IgE from patients sensitized to birch tree flower allergens. Thus, we preferred to use a homologous system; our positive reference pool contained 1,000 units of IgE for TES-Ag liter⁻¹. This unit was named the Toxocara unit (TU). The software reported OD260 of patient sera on the positive reference curve and converted them into TU liter⁻¹. The technical cutoff value was 1 TU liter⁻¹.

Statistical analysis. Statistical analysis was conducted by using an i386/387 computer with statistical software (Northwest Analytical, Portland, Oreg.). All the calculations were made with logarithmic values of data, so geometric means are given.

RESULTS

Serological examinations. Thirty-eight tests of the undiluted positive reference pool showed a mean OD310 of 0.992 (95% CI, 0.968, 1.016; range, 0.898 to 1.173; variation, 1%). At a 1:1,000 dilution, i.e., 1 TU, the mean OD420 of the positive reference pool was 0.050 (95% CI, 0.046, 0.054; range, 0.030 to 0.070; variation, 5%). The negative reference pool exhibited a mean OD420 of 0.003 (95% CI, 0.002, 0.004; range, 0.000 to 0.018; variation, 100%). Among sera from patients in group 1, 2, and 3, WB with TES-Ag yielded negative results (all groups) or displayed only high-molecular-weight banding patterns (group 3). The results of sIgE ELISA were presented in Table 1. Sera from groups 1 to 3 exhibited very low values, especially sera from allergic patients or those containing immune isohemagglutinins or heterophilic antibodies. Sera from patients in group 3 had the highest sIgE values, but the maximum value did not exceed 100 TU liter⁻¹.

Figure 1 shows sIgE results for group 4 patients. Sera from 131 of 150 (81.3%) patients with clinical toxocariasis were reactive in the sIgE-ELISA at ≥1 TU liter⁻¹, while sera from 138 (92%) patients were positive in the WB assay. A high proportion (18.7%) of sera from patients found to be positive by WB were negative by sIgE ELISA; sera from 12 patients (8%) presenting with a negative WB result displayed high titers for total IgE (range, 181 to 37,162 kIu liter⁻¹) and sIgE (range, 276 to 25,000 TU liter⁻¹).

An assessment of the efficacy of the sIgE ELISA for the follow-up of treated patients was carried out on the results for group 5 patients, as was the evaluation of the efficacy of DEC.

Results from statistical analysis. A study of the specificity and sensitivity of the sIgE ELISA was conducted on the results for patients in groups 1 to 4. Five cutoff values were tested: 1, 5, 10, 20, and 50 TU liter⁻¹. The first value corresponded to the technical threshold (Table 2).
The correlation of the sIgE ELISA with other parameters, i.e., clinical (duration of disease before consultation and clinical score) or biological (eosinophilia and total IgE level), was studied for the results for group 4 patients by using Spearman's rank test and multivariate regression analysis. No positive or negative correlation was found between sIgE values and duration of disease before consultation, clinical score, or eosinophil count (Spearman's coefficients values [0.052, 0.014, and −0.199] not significant). A strong correlation between sIgE and total IgE was found (Spearman's coefficient, 0.469; Z statistic, 6.16; P < 0.001).

In group 5 patients (therapeutic trial), at the control checkup significant variations were found for the clinical score (control group and treated patients), eosinophil count (control group and treated patients), total IgE (control group), and sIgE (treated patients) (Table 3).

### DISCUSSION

When it is used routinely and intensively, sIgE ELISA appears to be a simple and easy-to-use method. The use of undiluted sera avoided the tiresome procedure of mass dilutions and probably contributed to the excellent reproducibility of the method.

The intrinsic specificity of the sIgE ELISA was good, since this test reacted at a very low average level (mean, 2.1 TU liter⁻¹; 95% CI, 1.5, 2.9) with sera from allergic patients when the mean value of total IgE in the sera was high (912.4 kIU liter⁻¹; 95% CI, 555.1, 1,499.6). Non-specific adsorption of total IgE onto the polystyrol solid phase did not occur. When Oliver et al. (23) tested 58 serum samples from atopic or asthmatic patients that exhibited total IgE levels of ≥1,000 kIU liter⁻¹, they also found that the sIgE ELISA was non-reactive. Thus, the good statistical agreement found between sIgE and total IgE levels in patients in group 4 may be due to the marked stimulation of IgE synthesis that has been shown in many patients with helminthiasis, including toxocariasis (13, 15, 24).

In 1983, Smith et al. (27) demonstrated that TES-Ag contains blood group-like antigens; they stated that sera with immune isohemagglutinins could cross-react and thus yield false-positive results in the serodiagnosis of toxocariasis when sera were tested by TES ELISA (26). In 1985, Glickman and Schantz (12) showed that sera containing high titers of such haemagglutinins were not reactive when tested by TES ELISA, and we corroborated this point when we assessed the WB procedure for the serodiagnosis of toxocariasis (20). In the present study, neither sIgE ELISA was affected by such immune hemagglutinins.

Testing of sera from patients presenting with various helminthic diseases showed that the sIgE ELISA reacted at a low mean level (3.1 TU liter⁻¹; 95% CI, 2.6, 3.8), although 23.8% of these sera had ≥5 TU liter⁻¹. Those positivities could be due to concurrent minor toxocarial infestations, because that zoonosis is widespread. In a previous study, examination of sera from such patients by WB revealed that 6.7% (8 of 118 serum samples) displayed typical patterns (20).

Analysis of the results from group 4 patients, showed that a majority of the patients (52.1%) exhibited moderate values (from 1 to 100 TU liter⁻¹) and a high proportion of negative results (18.7%) was found. These two factors produced a moderate mean titer for the sIgE ELISA (49.8 TU liter⁻¹). Genchi et al. (9) have hypothesized that the synthesis of sIgE remains at a low level during the acute phase of toxocarial infection. Statistical analysis of data for group 4 patients, which indicated that there is no relationship between the sIgE level and parameters such as the estimated duration of the disease, the clinical score, and/or eosinophilia, does not agree with the hypothesis of Genchi et al. (9). The synthesis of sIgE for a given parasite is probably genetically regulated, although this point remains unclear (24).

The wide range of sIgE ELISA values in 138 patients with toxocariasis showing a positive WB result suggested that the two methods correlated poorly. Since the antigens that were transferred to the nitrocellulose sheet might be different from those that adhered to the ELISA plate, we studied the correlation between the detection of specific IgG by the TES ELISA and that by sIgE ELISA. TES ELISA values were available for 43 patients in group 3 and for 13 patients in group 4. These sera were tested by TES ELISA in a previous study (20). The results for the 43 serum samples from patients in group 3 showed a lack of correlation between the two methods (Spearman's coefficient, 0.19; Z statistic, 1.22). Among the 13 serum samples from patients in group 4, a reverse quantitative correlation was found (Spearman's coefficient, patients in −0.475; Z statistic, −1.634; P = 0.05).

Moreover, using the data published by Genchi et al. (9, 10), we assessed the quantitative correlation between the TES ELISA (IgG) and radioimmunoassay with TES-Ag (sIgE) for sera from 21 patients; Spearman's rank test was

### Table 3. Statistical analysis of the kinetics of various parameters in patients in group 5

<table>
<thead>
<tr>
<th>Consultation</th>
<th>Eosinophilia (cells μl⁻¹)</th>
<th>Total IgE (kIU liter⁻¹)</th>
<th>sIgE ELISA titer (TU liter⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEC-treated patients</td>
<td>Untreated patients</td>
<td>DEC-treated patients</td>
</tr>
<tr>
<td>First</td>
<td>1,005</td>
<td>803</td>
<td>574</td>
</tr>
<tr>
<td>Second</td>
<td>287</td>
<td>415</td>
<td>527</td>
</tr>
<tr>
<td>Wilcoxon's signed rank test</td>
<td>2.75 (P &lt; 0.001)</td>
<td>2.75 (P = 0.006)</td>
<td>NSb</td>
</tr>
</tbody>
</table>

* Values are means.

b NS, not significant.
not significant (Spearman’s coefficient, 0.078; Z statistic, 0.35).

From these findings it can be inferred that an assay that detects sIgE should not be used alone, but it appears to be a good complement to a method that detects IgG for TES-Ag. In fact, sera from 12 of 150 patients in group 4 were strongly positive for TES-Ag by sIgE ELISA only.

Prophylactic measures must be used in association with drug therapy for toxocariasis. When treated and control patients exhibited clinical and biological disturbances (on average, 4.6 months [95% CI, 3, 4.4] and 3.6 months [95% CI, 3.4, 7] after the presumed onset of disease, respectively), a significant decrease in the clinical scores and eosinophil counts was found in both groups 2 months after the first consultation. A remarkable point is the significant decrease in sIgE ELISA only in the DEC-treated group, indicating that for posttreatment follow-up assessment, the sIgE ELISA could be more valuable than ELISAs that detect IgG. The levels of specific IgG did not vary significantly, and this result is in agreement with the results of previous studies in two groups of children, in which Bass et al. (2) assessed the efficacy of thiabendazole versus that of placebo and checked the TES ELISA results 1 year later. Only a slight decrease in the average TES ELISA values was found in sera from the two groups.

For a long time it remained a question whether anthelmintics are effective for the treatment of toxocariasis. Previous reports describing the results of therapeutic trials with various benzimidazoles showed that these drugs did not affect the kinetics of biological parameters (2, 19, 28). In this study, the results for group 5 patients proved the efficacy of DEC, especially its effect on the sIgE ELISA level. Moreover, when the values of the parameters were compared among the two groups at the time of consultation, for controls, DEC was found to be more effective (P = 0.03 by Mann and Whitney’s one-tailed test; Z statistic, 1.9) on eosinophilia, a biological parameter that appears to be correlated with the presence of viable larvae in body tissues (29).

According to the results of this study, in the Laboratory of Parasitology the WB procedure remains the first-line assay for the serodiagnosis of human toxocariasis. A serum sample found to be negative for helminthic diseases by our panel of serodiagnostic tests and by WB is tested by sIgE ELISA. A discrepancy (i.e., negative WB and positive sIgE ELISA), if any, is interpreted in three ways when the patient’s case history is not available. When sIgE ELISA values range from 1 to 100 TU liter⁻¹, physicians are told to ask for repeated stool examination, including an examination by the Baerman’s method, plus other direct examinations when the patient lived in or was a native of a tropical country. When the sIgE titer lies between 100 and 500 TU liter⁻¹, a second serodiagnosis is carried out, when possible, 1 month later. Patient sera that exhibit titers of more than 500 TU liter⁻¹ are classified as toxocariasis cases.

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