Improved Primary Immunodiagnosis of Alveolar Echinococcosis in Humans by an Enzyme-Linked Immunosorbent Assay Using the Em2<sup>plus</sup> Antigen

BRUNO GOTTSTEIN,¹* PATRICK JACQUIER,² SOLANGE BRESSON-HADNI,³ AND JOHANNES ECKERT⁴

Institute of Parasitology, University of Berne, Länggassstrasse 122, CH-3012 Berne,¹ and Institute of Parasitology, University of Zürich, CH-8057 Zürich,² Switzerland, and Service d’Hépatologie, Centre Hospitalier Universitaire, F-25030 Besançon, France³

Received 3 September 1992/Accepted 19 November 1992

Alveolar echinococcosis (AE) in humans is generally a fatal disease when not diagnosed early enough to provide curative treatment such as radical surgery. Immunodiagnosis for early detection of AE was improved by the isolation of an affinity-purified metacestode Em2 antigen and by the synthesis of recombinant Echinococcus multilocularis antigen II/3-10. Both antigens were individually assessed by enzyme-linked immunosorbent assay (ELISA) and demonstrated high specificities and diagnostic sensitivities, although both missed approximately 4 to 11% of diagnostic cases of AE. To provide an optimal serodiagnostic test, we investigated the two purified antigens by using a test employing a mixture of both purified antigens (designated Em2<sup>plus</sup> antigen) in one assay. For comparative purposes, crude E. multilocularis and Echinococcus granulosus metacestode antigens were investigated as well. The Em2<sup>plus</sup> ELISA proved to be the optimal diagnostic test with the highest diagnostic sensitivity, 97%, in serum samples from 140 patients with AE and an overall specificity of 99% for infections due to other Echinococcus and non-Echinococcus parasites. The new test combination (Em2<sup>plus</sup> ELISA) is suggested for the serodiagnosis of AE in patients and for seroepidemiological surveys.

Alveolar echinococcosis is a mainly hepatic disease caused by infection with the metacestode (larval stage) of the tapeworm Echinococcus multilocularis, which occurs in foxes, dogs, cats, and other carnivores (1, 16). The distribution of E. multilocularis is geographically restricted to the northern hemisphere, where cases of alveolar echinococcosis in humans are diagnosed relatively frequently in central Europe, Russia, some areas of Asia (Siberia, China, and Japan), and western Alaska. In Switzerland for instance, 145 new cases of alveolar echinococcosis were diagnosed between 1970 and 1983 (3). Early serological diagnosis and subsequent treatment of persons with alveolar echinococcosis may reduce mortality (1, 9). In most laboratories, antibody detection in human sera is usually based upon the use of either crude Echinococcus granulosus or crude E. multilocularis antigen, both of which show various degrees of cross-reactivity and nonspecificity (6). These parameters were also demonstrated to be dependent on the origins of different parasite isolates (5). Together, these data have stimulated research directed towards the generation of purified antigens with improved operating immunodiagnostic characteristics. In this respect, two basically different E. multilocularis antigens which, however, have similar immunodiagnostic operating characteristics were demonstrated. One is the Em2 antigen (4) purified from E. multilocularis metacestode tissue extracts by affinity chromatography. The Em2 antigen has been widely evaluated and efficiently applied in enzyme-linked immunosorbent assays (ELISA) for primary clinical and seroepidemiological diagnoses (7, 9, 11, 12, 15) and for posttreatment follow-up studies (10, 13). Another highly antigenic polypeptide of E. multilocularis was produced by the recombinant-DNA technique with Escherichia coli (17, 18). The immunochemically purified recombinant E. multilocularis antigen (antigen II/3-10) demonstrated immunodiagnostic properties comparable to those of the Em2 ELISA: the antigens exhibited diagnostic sensitivities of 90 and 92%, respectively, and specificities for both antigens were 99%. Consequently, we designed a large study to directly investigate comparatively the two antigens for their diagnostic characteristics and also to evaluate a test system employing both antigens simultaneously in the same test procedure (double-antigen ELISA, hereafter referred to as Em2<sup>plus</sup> ELISA). Furthermore, we assessed comparatively two crude E. multilocularis metacestode extract antigens and a crude E. granulosus hydatid cyst fluid antigen and the potential of combining Em2<sup>plus</sup> ELISA with E. granulosus hydatid fluid (EgHF) ELISA for a correct differential diagnosis between cystic and alveolar echinococcoses.

MATERIALS AND METHODS

Serum samples. The serum samples used for assessing diagnostic specificities of the different ELISA antigens were from 140 patients with active hepatic alveolar echinococcosis. Sera from Alaskan, French, and Swiss patients were uniquely diagnostic and were sampled prior to any therapeutic intervention; the corresponding information could not be obtained for the Japanese-patient sera. All diagnoses of alveolar echinococcosis had been confirmed by clinical, histological, and serological methods (1). Geographical origins of patients included in the study were as follows: Switzerland, 53; France, 52; Alaska, 13; Japan, 22.

The serum samples used for assessing test specificities were obtained from patients with clinically, parasitologically, and/or histologically proven infections involving the following parasite species (disease name and number of investigated patients are in parentheses): E. granulosus...
(cystic echinococcosis, 124); Fasciola hepatica (fascioliasis, 20); Schistosoma spp. (schistosomiasis, 17); Taenia solium (cysticercosis, 20); Taenia saginata (taeniasis, 17); Ascaris lumbricoides (ascariasis, 10); Strongyloides stercoralis (strongyloidiasis, 10); Toxocara canis (visceral larva migrans, 20); Trichinella spiralis (trichinosis, 20); Onchocerca volvulus (onchocerciasis, 10); Plasmodium falciparum (malaria, 10); Toxoplasma gondii (toxoplasmosis, 10), and Entamoeba histolytica (amebiasis, 20). All of these sera had been previously selected on the basis of positivity in the demonstration of antibodies against antigens of the respective parasite species. They were partly the same as those employed for a similar purpose in a study on toxocariasis (14).

The serum samples used for assessing potential nonspecific reactions related to other hepatic diseases were obtained from patients (number of patients in parentheses) with viral hepatitis (12), carcinoma (11), alcoholic cirrhosis (6), and primary biliary cirrhosis (15), and the serum samples used for assessing potential nonspecific reactions related to systemic disorders included samples obtained from patients (n) with myeloma (10), collagenosis (20), and rheumatoid arthritis (10) and eight samples with antinuclear antibodies.

The sera used for the determination of normal ranges and parameters for the different types of antigens were from 500 healthy Swiss blood donors, matched by age (range, 20 to 69 years) within 10-year intervals and by sex. The same donors were employed for a similar purpose in a study on toxocariasis (14).

ELISA. Antigen-specific serum immunoglobulin G was detected by ELISA. All test serum samples had been kept frozen (−80°C) and were examined at the same time and under uniform conditions in order to avoid intertrial variations. The ELISA was performed basically as described previously (4, 7, 17). The following antigens were included in the study: an affinity-purified species-specific antigen (Em2 antigen) derived from E. multilocularis (4), a recombinant E. multilocularis antigen (II/3-10 antigen) purified by Müller et al. (17), crude E. multilocularis antigens extracted from metacercade tissue according to the method of Gottstein et al. (7) (CH-10 and N3C antigens from a Swiss and a French E. multilocularis isolate, respectively), and a crude E. granulosus hydatid cyst (camel origin) fluid antigen (EgHF) (8). Polystyrene surfaces of microELISA plates (Immuno-plates I-96F; Nunc, Roskilde, Denmark) were sensitized with antigens at the following concentrations: Em2 antigen, 4 μg/ml; II/3-10 antigen 2 μg/ml; CH10, N3C, and EgHF antigens, 10 μg/ml each. Em2Plus antigen consisted of Em2 and II/3-10 antigens mixed at the concentrations listed above. Other technical parameters were as follows: serum dilutions were 1:200; the conjugate was alkaline phosphatase-labelled anti-human immunoglobulin G (specific for Fc fragment; Dako, Copenhagen, Denmark); the substrate was 4-nitrophenylphosphate; the reaction was stopped with 3 N NaOH; and optical measurements were made at A405 by using a Dynatech M600 reader coupled to a Macintosh SE computer with Immunosoft software from Dynatech. Elementary test parameters were established for each antigen by investigating serum samples from 500 healthy Swiss blood donors, and each assay was referenced by including a standard positive serum sample with defined anti-Em2 antibody activity (19) and appropriate negative and additional positive (19) reference serum samples. Threshold values discriminating between diagnostically positive and negative reactions were thus determined for each antigen listed below by using the criteria for interpretation of seropositivity reported earlier (7). The results are expressed in arbitrarily defined antibody units (AU), which reflect a percent relation of test serum sample reactivity to the antibody activity of the positive reference serum sample (the reference serum sample was arbitrarily set at 100 AU, corresponding to 100% reference antibody reactivity and simultaneously to the A405 value measured for this sample). Samples were classified as negative when antibodies could not be detected (AU = 0).

Reproducibility of results was monitored by including on each microELISA plate a negative serum sample in triplicate and three positive alveolar echinococcosis serum samples with defined (19) anti-Em2 antibody activities (serum samples A to C [100, 65, and 35 AU, respectively]), with each sample used in triplicate.

For assessing the potential to perform an optimal differential diagnosis between cystic and alveolar echinococcoses by using the Em2Plus ELISA and the EgHF ELISA, a mathematical approach similar to the one described earlier was performed (7). The formula was arbitrarily modified for easier presentation and interpretation of an optimized differentiation coefficient: (AU in Em2Plus ELISA/AU in EgHF ELISA) x 8.

By using this formula, all serum samples with a differential coefficient of ≤1 were attributed to the E. multilocularis infection (alveolar echinococcosis), whereas those with coefficients of >1 were attributed to the E. granulosus infection (cystic echinococcosis).

RESULTS

Diagnostic sensitivities for the different antigens and patients with alveolar echinococcosis (grouped according to their geographical origin) are listed in Table 1. Generally, all antigens exhibited relatively high diagnostic sensitivities. For crude E. multilocularis antigens, it ranged, depending upon the geographical origin of the patient, between 91 and 100%. The range of diagnostic sensitivities for the Em2 ELISA varied around 92% for European and Alaskan-patient serum samples; it was markedly lower (77%) for Japanese-patient serum samples. Antibodies to the recombinant II/3-10 antigen were detectable in 82 to 92% of Japanese- or European-patient serum samples but only in 62% of Alaskan-patient serum samples. Remarkably, all losses in diagnostic sensitivity resulting from the use of purified E. multilocularis antigens were reverted and corrected by the combination of the two antigens in question (Em2Plus ELISA) in that all diagnostic sensitivities for different geographical areas were increased to at least 96% (range, 96 to 100%). The optimal overall diagnostic sensitivity was matched by the crude EgHF and the combined purified Em2Plus antigen, both scoring 97.1%.

Table 2 lists individually the specificities for the different antigens. The specificities shown in Table 2 were determined by using serum samples from patients with clinically and/or parasitologically proven parasites, all sera having previously shown antibody activities with crude antigens from the respective infecting parasite species. With regard to genus specificity (i.e., cross-reactions due to infection with the closely related E. granulosus), the Em2 antigen and the combination of both antigens (Em2Plus) had unambiguously the highest specificity (94%), with the recombinant antigen, II/3-10, having the next highest specificity (74%). Crude E. multilocularis antigens showed extensive cross-reactivity with E. granulosus-infected serum samples.

Similar findings concerning the overall specificity related to infection with other helminth or protozoan parasite spe-
TABLE 1. Diagnostic sensitivities of crude, purified, and recombinant *Echinococcus* antigens for immunodiagnosis of alveolar echinococcosis by ELISA in patients with confirmed alveolar echinococcosis

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Sensitivity (%) of serum samples from:</th>
<th>No. of positive serum samples</th>
<th>Overall diagnostic sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Switzerland (n = 53)</td>
<td>France (n = 52)</td>
<td>Alaska (n = 13)</td>
</tr>
<tr>
<td>CH-10</td>
<td>94 (50)</td>
<td>96 (50)</td>
<td>92 (12)</td>
</tr>
<tr>
<td>N3C</td>
<td>98 (52)</td>
<td>96 (50)</td>
<td>92 (12)</td>
</tr>
<tr>
<td>Em2</td>
<td>90 (48)</td>
<td>92 (48)</td>
<td>92 (12)</td>
</tr>
<tr>
<td>II/3-10</td>
<td>92 (49)</td>
<td>88 (46)</td>
<td>62 (8)</td>
</tr>
<tr>
<td>Em2plus</td>
<td>96 (51)</td>
<td>96 (50)</td>
<td>100 (13)</td>
</tr>
<tr>
<td>EgHF</td>
<td>100 (53)</td>
<td>98 (51)</td>
<td>85 (11)</td>
</tr>
</tbody>
</table>

* The number of positive samples is given in parentheses.


described earlier for a *Toxocara* ELISA (14) and, thus, considered acceptable.

With regard to the assessment of an optimal differential diagnosis between cystic and alveolar echinococcoses by calculating a differential coefficient, the following results were obtained. From a total of 264 serum samples from patients with echinococcosis which were investigated in the present study, 258 serum samples showed reactivity with either one or two of the antigens (EgHF or Em2plus). The differential coefficient was calculated for these initially seropositive sera, and the procedure resulted in the following correct diagnoses: for infections with *E. multilocularis*, a correct differential diagnosis (differential coefficient ≥1) was obtained in 91.9% of the cases, and for infections with *E. granulosus*, a correct differential diagnosis (differential coefficient <1) was obtained in 96.8% of the cases. The overall rate of correct differential diagnosis was, consequently, 93.2%.

**DISCUSSION**

Immunodiagnosis of alveolar echinococcosis is doubtlessly one of the most important diagnostic tools in early detection of the infection. Early detection of infection improves prognosis for the patient in question in that surgical treatment becomes more likely to be radical in removal of the entire parasite. The use of crude or fractionated *E. multilocularis* metacysteate antigens in ELISA had already demonstrated improved immunodiagnostic characteristics compared with the widely used EgHF antigen in that the

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. of samples positive by ELISA*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. granulosus</em> (hydatidosis) (n = 124)*</td>
</tr>
<tr>
<td></td>
<td>Trematodes</td>
</tr>
<tr>
<td></td>
<td>Fasciolasis (n = 20)</td>
</tr>
<tr>
<td>CH-10</td>
<td>77 (38.9)</td>
</tr>
<tr>
<td>N3C</td>
<td>102 (17.7)</td>
</tr>
<tr>
<td>Em2</td>
<td>7 (94.4)</td>
</tr>
<tr>
<td>II/3-10</td>
<td>8 (93.5)</td>
</tr>
<tr>
<td>Em2plus</td>
<td>32 (74.2)</td>
</tr>
<tr>
<td>EgHF</td>
<td>124*</td>
</tr>
</tbody>
</table>

* Of the 40 samples from patients with protozoan infections (malaria [n = 10], toxoplasmosis [n = 10], and amebiasis [n = 20]), none were positive by ELISA.

a Percent specificity is given in parentheses.

b Diseases (n): ascariasis (10), strongyloidiasis (10), toxocariasis (20), trichinosis (20), and onchocerciasis (10).
diagnostic sensitivity for alveolar echinococcosis was markedly increased (4, 7). As an alternative to the use of nonstandardized crude *E. multilocularis* antigens, the isolation and production of purified *E. multilocularis* antigens were suggested to obtain optimized immunodiagnostic properties. In this respect, both the Em2 and the recombinant *E. multilocularis* II/3-10 antigen had shown excellent specificities, with a minor loss in diagnostic sensitivity (4, 17). We have now shown that the simultaneous use of the two antigens in question (Em2plus antigen) exhibited optimal immunodiagnostic operating characteristics. A special feature of the present investigation is the demonstration that the few patients without anti-Em2 antibodies all had anti-II/3-10 antibodies, and conversely, II/3-10-negative patients were all Em2 positive. This observation was of special significance for patients from geographical areas with overall lower diagnostic specificities for the individual purified antigens, such as Japan for the Em2 antigen and Alaska for the recombinant II/3-10 antigen. Combined with an *E. granulosus* antigen such as in the EgHF ELISA, the Em2plus ELISA allows an accurate differential diagnosis between cystic and alveolar echinococcoses (differentiation rate, 93.2%) by calculating a differential coefficient. The present method fits that described earlier (7) but uses now an arbitrarily modified formula for easier presentation and interpretation of results.

The comparative combination of the diagnostic specificities and relative specificities listed above clearly shows that the most accurate diagnostic tool for immunodiagnosis of alveolar echinococcosis is represented by the use of the Em2plus antigen combination (Em2plus ELISA). The diagnostic characteristics include both an optimal diagnostic sensitivity and a very low degree of cross-reactivity. Therefore, an assay based upon the simultaneous use of these antigens is presently being commercialized. The test will furthermore be evaluated for its additional applicability in follow-up studies after surgery including cases of liver transplantation (2) or during and/or after chemotherapy, as has already been performed for the Em2 ELISA (10, 13, 15). On the basis of the present results, we propose as a general strategy for immunodiagnosis of echinococcosis to combine the Em2plus ELISA with an EgHF ELISA for obtaining optimal diagnostic test sensitivity for alveolar and cystic echinococcoses; specificity under these circumstances will be optimal for alveolar echinococcosis, whereas the problems of specificity related to EgHF ELISA (and any other related *E. granulosus* test) still need further improvement.

ACKNOWLEDGMENT

We thank I. Tanner for excellent technical assistance.

REFERENCES