Immunological Diagnosis of Human Hydatid Cyst Relapse: Utility of the Enzyme-Linked Immunoelectrotransfer Blot and Discriminant Analysis

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A discriminant technique was applied to the different serological patterns obtained by enzyme-linked immunoelectrotransfer blotting (EITB) and by conventional immunological tests, in order to differentiate the residual antibody patterns present in healed hydatidosis from the ones present in patients with active hydatidosis. For this purpose, specific antibodies against Echinococcus granulosus were detected by indirect hemagglutination, agglutination of latex particles, basophil degranulation, and EITB for 23 patients with active hydatidosis and 45 patients with surgically cured hydatidosis. Discriminant analysis of the different serological patterns obtained by EITB and conventional serology correctly classified 92.54% of patients (93.3% if the patients are differentiated according to the time elapsed since surgery). This method detected the presence of active hydatidosis in 95.6% of patients for whom abdominal ultrasonography had confirmed the presence of active hydatid cysts. The global specificity was 88.9%. The specificity was 97.1% for patients who had been operated on 3 years ago or more and 63.6% for patients with less time since surgery.

Hydatidosis is a disease caused by the larval stages of several cestodes belonging to the Echinococcus genus, and diagnosis is still an unresolved problem (15, 17). Echinococcus granulosus has a very complex antigenic structure, and hydatid disease has a slowly developing course, but there is no immunological test with 100% sensitivity to detect antibodies against the parasite. In different series of patients, from 3 to 40% of human hydatidosis cases are found to be seronegative (5). These negative reactions are more frequent when the cysts are located in the lung and brain or in those cysts with hyaline or calcified walls (3, 16).

The “gold standard” immunological test would be one which could detect early on the complete resolution of the disease after medical or surgical treatment. Many attempts have been made to assess complete resolution of the disease after adequate therapy by immunological methods. Detection of serum antibodies, circulating antigen, and circulating immune complexes has been reported to be of potential use in monitoring cystic echinococcosis patients after surgical and chemotherapeutic treatments. Classical immunological tests can be positive for a long time after the surgical eradication of all of the hydatid cysts. Only when a progressive decrease in antibody titers could be detected would the supposition of complete healing appear to be more feasible. Complement fixation and indirect immunofluorescence are the classical tests that more quickly become negative after complete resolution of hydatid cysts, but the results are inconsistent, and, in different revisions, there have been positive results with these immunological tests more than 10 years after curative treatment (4, 7, 12, 14, 22, 23, 28–30, 32).

The main subject of this paper is the serological pattern obtained by enzyme-linked immunoelectrotransfer blotting (EITB) in order to improve the sensitivity of this procedure for the diagnosis of hydatid cyst relapse, differentiating the serological patterns of patients with active hydatid disease from those of patients who had had hydatid cysts but who were successfully treated by surgical methods. Second, the effect of the time elapsed after surgery on the accuracy of the prediction of the relapses by serological patterns was studied. For these purposes, a discriminant analysis of the bands obtained by EITB, a sensitive and specific method to diagnose hydatid disease (8, 13, 14, 25, 27, 31), and of conventional serology results was performed in order to detect relapses with a good sensitivity and specificity. The usefulness of this procedure to enhance EITB resolution in the diagnosis of hydatid disease has been described earlier (8).

MATERIALS AND METHODS

Patients studied. Sixty-eight patients were included in the study and were separated into the following groups. Group 1 consisted of 25 patients with active hydatid cysts. Twenty-two of them had fertile hepatic cysts. One patient had a meningeal relapse. Sixteen patients had newly diagnosed cysts, and seven corresponded to postsurgical relapses. Group 2 consisted of 45 patients with a past history of hydatidosis who were cured by surgical treatment and who did not have any sign of active hydatidosis at the time of the study. Thirty-four of them had been treated 3 years or more before this study (group 2.1), and 11 had been treated within the previous 3 years (group 2.2).

Sensitivity and specificity studies of each test, conventional or EITB, for the diagnosis of hydatid disease, including control sera of patients without any history of hydatid disease and sera from patients with parasitosis other than hydatidosis, had been performed and published earlier (8).

For all patients a chest X ray and abdominal ultrasonography were performed, as were conventional tests for the serological diagnosis of hydatidosis, i.e., indirect hemagglutination (IHA) and latex agglutination (LA). The basophil degranulation (BD) test was performed for 22 patients in group 1 and for the 45 patients in group 2. BD could not be performed for the patient with a meningeal relapse due to technical problems.

None of the patients received antihelmintic drugs, and those whose cysts had calcified cyst walls were not included in the study.

Serum samples from all of the patients described above were stored at –40°C. Complete blood samples, anticoagulated with lithium heparin, were drawn from 67 of the patients for immediate performance of the BD test. IHA test. The commercial Cellognost Echinococcosis (Behring Diagnostics GmbH, Marburg, Germany) test was used. Results equal to or higher than 1:64
were considered positive when hemagglutinins against type O erythrocytes were absent.

**LA test.** The commercial Agglutinotest Echinococcosis (Ismunit Woerden Netherlands) test was employed according to the manufacturer's recommendations, and sera whose titers were equal to or greater than 1:2 were considered positive.

**BD test.** The BD test was performed by the method of Mir et al. (18).

### Immunolectrotransfer performance

Hydatid cyst fluid from a human fertile hepatic cyst was obtained by sterile puncture during surgery and was processed as described earlier (18). Briefly, hydatid fluid was centrifuged at 900 × g for 15 min, and the supernatant was sterilized by filtration through a Millipore filter (pore diameter, 0.45 μm). Thereafter, it was dialyzed in phosphate-buffered saline at 4°C for 24 h and kept at −10°C.

Two tests were standardized for the detection of immunoglobulin G antibodies against the different antigens present in hydatid fluid by the previously described method (6). The first one was performed with unreacted antigens, and the other was performed with antigens previously reduced with 2-mercaptoethanol in order to destroy disulfide bonds.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of 1 mm in thickness and 17 cm in width with continuous gradients of 5 to 20% acrylamide were used; these were prepared by standard methods (6, 8–10, 30).

**EITB** was performed as described previously (8). Samples consisting of 1.5 ml of dialyzed hydatid fluid with concentrations of 0.75 ml of sample buffer (62 mM Tris-HCl [pH 6.8], 2% [wt/vol] sodium dodecyl sulfate, 10% [vol/vol] glycerol, 0.00125% [wt/vol] bromophenol blue). The final protein concentration was between 60 and 100 μg/ml. The mixture was denatured in a boiling water bath. 2-Mercaptoethanol (5% [vol/vol]) was added to the sample buffer for the test with reduced antigens. Electrophoresis and electrotransfer were performed under standard conditions.

In order to calculate the molecular masses of the different proteic bands, the method of Plitkay et al. (21) was used, with high- and low-molecular-mass markers which were separated in the same gel of a hydatid fluid sample, electrotransferred to the same sheet, and dyed with India ink (11). The sheets were cut into 3-mm-wide strips, and a standard procedure for the immunoenzymatic test was followed (8, 9, 30).

In all EITBs (up to 15), a positive serum, belonging to a patient with multiple peritoneal hydatidosis with an IHA level of greater than 1:4,096, and a negative serum sample, from an 11-month-old child without a clinical history of hydatidosis, were included. Only the reactive bands which consistently appeared in the tests performed with the positive control serum were used for the discriminant analysis. A band with an approximate molecular mass of 170 kDa which appeared in all strips with nonreduced antigens and another one with an approximate molecular mass of 70 kDa which appeared in the reduced strips were discarded for the statistical analysis, because it is known that they correspond to the presence of human immunoglobulin G in the hydatid fluid.

### Statistical analysis

The presence or absence of any specific reactive band and the results of conventional serology considered in a qualitative manner were used to build a polynomial equation named the discriminant function by using the minimization of the Wilks λ method (1, 24, 26). An extensive description of the statistical method used was reported previously (8). The function built in this way, and applied to each patient, yielded an individual value named the discriminant score which allowed the calculation of the probability of having an active disease, treated and cured. The statistical method used was reported previously (8). The function built in this way, and applied to each patient, yielded an individual value named the discriminant score which allowed the calculation of the probability of having an active disease, treated and cured.

The linear function which best discriminated the patients belonging to group 1 (with active hydatid cysts) and the patients belonging to group 2 (with past history of hydatid disease, treated and cured) was DS = 0.6358(X1) + 3.6153(X2) + 0.5046(X18) − 2.3446, where DS is the discriminating score for each patient and X1, X2, and X18 are 1 or 0, according to the criteria described above.

The mean values of the discriminating score (centroids) for each group were 2.3882 for patients with active hydatid cysts and −1.1676 for patients without hydatid cysts. A patient was classified in group 1 (with active hydatid disease) if the discriminant score for this patient was greater than or equal to 2.3882; all other patients were classified in group 2 (with past history of hydatid disease, treated and cured).

### Table 1. Results obtained with conventional serology

| Group or subgroup | IHA | BD | LA |
|------------------|-----|----|----|
| 1                | 100 | 95.4 | 73.91 |
| 2                | 88.89 | 46.67 | 33.33 |
| 2.1              | 91.17 | 41.18 | 35.29 |
| 2.2              | 81.81 | 63.63 | 27.27 |

* Group 1, patients with active disease; group 2, treated and cured patients; subgroup 2.1, patients treated 3 years or more before; subgroup 2.2, patients treated within the previous 3 years.

### RESULTS

**Conventional serology.** Percentages of positive results obtained with conventional serology are shown in Table 1. IHA was not a good method for the postsurgery surveillance of hydatid disease, as it remained positive in 91% of patients with proved hydatid disease 3 years after effective surgical treatment. BD and LA had better results but remained positive in 41% and 35% of the cured patients 3 or more years after surgical treatment, respectively.

**EITB.** The bands obtained by EITB with or without 2-mercaptoethanol treatment for the two groups of patients studied are shown in Fig. 1; only the bands which appeared in all of the tests performed with the same positive control serum are marked, and they are the only ones which were used in the statistical analysis. In order to facilitate the statistical analysis of the results, the different bands were given separate designations. Bands obtained when the hydatid fluid was treated with 2-mercaptoethanol were designated as follows: X1, the two bands of more than 200 kDa; X2, band of 145 kDa; X3, band of 36 kDa; X4, band of 33 kDa; X5, band of 20 kDa; X6, band of 17 kDa; and X7, band of 9 kDa. Bands obtained when the hydatid fluid was treated with sample buffer without 2-mercaptoethanol were designated as follows: X8, band of more than 200 kDa; X9, band of 70 kDa; X10, band of 60 kDa; X11, band of 50 kDa; X12, band of 32 kDa; X13, band of 21 kDa; X14, band of 16 kDa; and X15, band of 9 kDa.

The relative frequencies of IgG antibodies against *E. granulosus* antigens obtained with EITB are shown in Table 2.

For the discriminant analysis, all of the bands shown in Table 2 were considered. The results obtained with conventional serology, considered in a qualitative manner, were also included and are cited as follows: IHA, X16; LA, X17; and BD, X18. The linear function which best discriminated the patients belonging to group 1 (with active hydatid cysts) and the patients belonging to group 2 (with past history of hydatid disease, treated and cured) was DS = 0.6358(X1) + 3.6153(X2) + 0.5046(X18) − 2.3446, where DS is the discriminating score for each patient and X1, X2, and X18 are 1 or 0, according to the criteria described above.

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**FIG. 1.** Description of the different bands detected by EITB. Lanes 1 to 4, strips obtained with sera from two patients with hydatid cysts treated with surgery 2 years (lanes 1 and 2) and 5 years (lanes 3 and 4) before and without symptoms. Lanes 5 to 8, strips obtained with sera from two patients with hydatid cyst relapse 3 years (lanes 5 and 6) and 4 years (lanes 7 and 8) after surgery. Lanes 1, 3, 5, and 7, with reduced antigens; lanes 2, 4, 6, and 8, strips with unreduced antigens. R, molecular masses for reduced antigens; UR, molecular masses for unreduced antigens.
The discriminant function was DS = 2.08(X9) + 2.04(X4) – 1.77(X14) – 1.11(X9) + 9.21(X10) + 1.02(X19) – 5.1. The centroids (mean values of discriminant scores for each group) were 6.1712 for group 1.1 (X8) was the most useful marker of active disease when it was included; on the other hand, it remained positive in 89% of the patients treated and cured. BD had the best results of the three conventional tests. It was positive in 95.4% of patients with active cysts and negative in 53.3% of patients in group 2 (treated and cured); the percentage of negatives rose to 58.8% in group 2.1 (patients treated more than 3 years before). BD was the only conventional test that was included in the discriminant functions. BD results did not affect the specificity of the predictions, and they only had little effect on the final probability values.

EITB coupled to a discriminant analysis had greater power to differentiate residual antibody patterns of cured hydatidosis from those of active hydatid disease. The results were much better when EITB was applied to a population that had been surgically treated 3 years or more before the study. In this case, the negative predictive value was 95.7%. When the patient had been surgically treated less than 3 years before, the antibody patterns were less distinctive, and the predictive value of a positive result was 95.7%. When the patient had been surgically treated less than 3 years before, the antibody patterns were less distinctive, and the predictive value of a positive result was 95.7%. When the patient had been surgically treated less than 3 years before, the antibody patterns were less distinctive, and the predictive value of a positive result was 95.7%.

A comparative study between a group of patients with active hydatid disease and a group of patients with cured hydatid disease with various times of evolution has been performed. We realize that the most accurate method to study the serological patterns of relapsed hydatid disease would be to compare the serological patterns of patients with cured hydatid disease with those of patients with relapses of hydatid disease or, to perform better, a prospective study of the serological evolution of a significant number of patients with hydatid disease. This accurate study could not be performed because we did not have enough patients with hydatid relapse. Therefore, the patients with hydatid relapse and the patients with untreated and active hydatid cysts were considered to represent similar situations and were put in the same group. Hydatid cysts of lungs were excluded because they do not represent a situation comparable to relapsed cysts. On the other hand, the serological patterns obtained from the patients with postsurgical relapse were quite similar to the ones obtained from patients with new hydatid cysts.

The results obtained with the conventional serological tests were comparable to those reported in other series (2, 14, 17, 20, 29). As was expected, no conventional test allowed an accurate differentiation between active and cured hydatid disease. LA was negative in 33.3% of patients in group 2 (treated and cured), but it was positive in only 73.9% of patients with active disease. IHA detected all active cysts because only active hydatid cysts from the liver and one disseminated relapse were included; on the other hand, it remained positive in 89% of the patients treated and cured. BD had the best results of the three conventional tests. It was positive in 95.4% of patients with active cysts and negative in 53.3% of patients in group 2 (treated and cured); the percentage of negatives rose to 58.8% in group 2.1 (patients treated more than 3 years before). BD was the only conventional test that was included in the discriminant functions. BD results did not affect the specificity of the predictions, and they only had little effect on the final probability values.

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As may be seen in the three discriminant functions built, the presence of antibodies against the 70-kDa unreduced protein (X9) was the most useful marker of active disease when it was considered separately. It was negative only in the patient with meningeal relapse due to an arterial dissemination through...
aorta invasion of a giant hepatic cyst, which caused him to be erroneously diagnosed in all discriminant analyses. Hydatid cyst rupture is associated with the presence of circulating antigen and immune complexes (33); therefore, the intra-aortic disruption of the hydatid cyst was probably correlated with the presence of circulating antigen, a known cause of false-negative results in antibody assays (5), and it could explain the clearance of certain EITB bands which would be forming immune complexes.

Thus, in patients surgically treated 3 years before or more, the presence of antibodies against the 70-kDa unreduced pro-immune complexes.

clearance of certain EITB bands which would be forming immune complexes.

It is evident that the discriminant functions obtained in this study are the best ones for classifying the patients for whom they were constructed; therefore, with the general population, the predictive values will presumably be lower. On the other hand, better discriminant functions would be constructed if EITB were applied to more patients.

In spite of the hopeful results, we cannot conclude that the morphological methods for the surveillance of hydatidosis may be avoided. Broader studies will probably give better discriminant functions for the early diagnosis of hydatid relapse by immunological methods.

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