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Serological Differentiation between Cystic and Alveolar Echinococcosis by Use of Recombinant Larval Antigens

MATTHIAS HELBIG,[1] PETRA FROSCH,[1] PETER KERN,[2] AND MATTHIAS FROSCH[1]*

Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, 30623 Hannover,[1] and Medizinische Universitätsklinik und Poliklinik Ulm, Sektion Infektiologie und Klinische Immunologie, 89081 Ulm,[2] Germany

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Two recombinant antigens of the larval stages of *Echinococcus granulosus* and *Echinococcus multilocularis*, termed EG55 and EM10, respectively, were applied for serodiagnosis and serological differentiation between parasitic infections caused by the metacestode tissue of both tapeworms. Antigen EM10 is synthesized by *E. multilocularis* larvae. Antigen EG55 represents the recombinant form of the low-molecular-weight subunit of antigen B, which is an *Echinococcus* genus-specific antigen. Both recombinant antigens were expressed as glutathione *S*-transferase fusion proteins. A sandwich enzyme-linked immunosorbent assay with monoclonal antibodies against EM10 and EG55 as capture reagents for the recombinant antigens was established and was evaluated with 74 serum samples from patients with histologically confirmed alveolar echinococcosis and 63 serum samples from patients with histologically confirmed cystic echinococcosis. A sensitivity of 93.2% and a specificity of 96.8% were achieved for the serodiagnosis of alveolar echinococcosis. Cystic echinococcosis could be detected with a sensitivity of 89.1% and a specificity of 98.6%.

Alveolar echinococcosis, which is caused by the larval stage of the tapeworm *Echinococcus multilocularis*, is a life-threatening parasitosis in countries of the Northern Hemisphere. Human infections occur by ingestion of eggs excreted with the feces of infected foxes, dogs, or cats. The metacestodes of the tapeworm develop mainly in the liver of their intermediate host and form an infiltrative growing tumor. In contrast, the metacestodes of the closely related tapeworm *Echinococcus granulosus*, which occurs worldwide, especially in countries with low hygienic standards, grow as large hydatid cysts by internal budding of brood capsules (13). Since both parasitic infections are lethal without treatment, an early diagnosis of both echinococcal diseases is essential for curing these parasitoses by chemotherapy and/or surgery. Interpretation of results of diagnostic methods like computer tomography or sonography is often difficult. Therefore, radiological techniques must be combined with serological assays. The parasitoses cannot be differentiated unambiguously by use of crude echinococcal antigen extracts of *E. multilocularis* larvae or of the hydatid fluid from *E. granulosus* cysts, and sera from patients infected with other helminthic parasites may also exhibit cross-reactivities (4, 13, 19, 28, 36). Furthermore, the antigenic composition of the hydatid fluids from naturally infected animals may be variable (15, 27, 30), which hinders standardization of assay systems based on this parasitic material.

Recent reports focused on the identification and purification of species-specific echinococcal antigens (1, 12, 20). By immunoblotting it was demonstrated that a high percentage of patients suffering from cystic echinococcosis showed a characteristic antigen reactivity pattern (21, 32, 34). The most prominent antigen in the hydatid fluid of *E. granulosus* is antigen B, which, however, is an *Echinococcus* genus-specific antigen (5, 26). The small subunit of antigen B migrates as a 12-kDa antigen in sodium dodecyl sulfate-polyacrylamide gels (31). In different studies, antibodies against antigen B were detected in the sera of 77 to 91% of *E. granulosus*-infected patients and in the sera of 39% of patients suffering from alveolar echinococcosis (23, 24, 31). For the serodiagnosis of an *E. multilocularis* disease, Gottstein (12) described the *E. multilocularis* metacestode-specific antigen Em2a, which was purified by affinity chromatography. In 95% of cases of disease, alveolar echinococcosis could be differentiated by use of Em2a (14). However, isolation of antigens from metacestode tissues requires cultivation of the echinococcal larvae in laboratory animals. Alternatively, synthetic peptides (3, 7) and recombinant antigens (10, 11, 17, 18, 25, 30, 35) were defined for the serodiagnosis of echinococcal disease. Here, we describe the use of recombinant antigen EM10, an *E. multilocularis*-specific antigen (10), and the recombinant low-molecular-weight subunit of antigen B for serological differentiation between *E. multilocularis* and *E. granulosus* infections.

MATERIALS AND METHODS

Isolation and characterization of the recombinant antigens used in the study. Antigen EM10 is synthesized in the metacestode tissue of *E. multilocularis*. Preliminary experiments demonstrated a high sensitivity and specificity of recombinant antigen EM10 for the serodiagnosis of alveolar echinococcosis (10). An *Escherichia coli* clone expressing the low-molecular-weight subunit of antigen B was isolated from a cDNA expression library of the larval stage of *E. granulosus* by immunoscreening with pooled sera obtained from *E. granulosus*-infected patients. For construction of the cDNA library, 20 μg of total RNA was isolated from the larvae of a cyst of human origin. After purification of 250 ng of mRNA by using oligo(dT) spun columns (Pharmacia Biosystems, Freiburg im Breisgau, Germany), cDNA was synthesized as described previously (37). First-strand synthesis was performed with an oligo(dT) primer that included an internal *Xho*I site at the 5′ end [5′-$(GA)_{10}$AAGTAGTCTC GAG$(T)_{15}$-3′] and Moloney murine leukemia virus reverse

* Corresponding author.

transcriptase (Stratagene, Heidelberg, Germany). The first strand was tailed with guanidine by the addition of [α-^{32}P] dGTP and terminal transferase (Pharmacia Biosystems). Second-strand synthesis was performed with a polyC primer [5′-TGTTTCCTGTCTACAGTGT$(C)_{15}$-3′] and *Taq* DNA polymerase. Amplification of the cDNA by polymerase chain reaction (PCR) was performed by standard protocols (29) with the primers 5′-TGTTTCCTGTCTACAGTGTC-3′ and 5′-$(GA)_{10}$AAGTAGTCTCGAG-3′. Cloning of the amplified cDNA into the λZAPII vector, expression of the recombinant antigens in *E. coli* SURE cells (Stratagene), and immunoscreening with patient sera were performed as described previously (11). One immunoreactive clone of this library, termed EG55, was sequenced. A 95.7% nucleotide sequence identity and a 85% amino acid sequence identity to the already published, incomplete sequence of antigen B were observed (30). In addition to the published sequence, clone EG55 synthesized an additional 28 amino acids at the N terminus, including 16 amino acids which showed characteristics of eukaryotic signal sequences (data not shown) (9).

Synthesis and purification of GST fusion proteins. For expression of fusion proteins, the coding sequence of EM10 was amplified by PCR (10) and was cloned in frame into the *Sma*I and *Eco*RI sites of the expression vector pGEX-3X (Pharmacia Biosystems) (33). For subcloning of the antigen B subunit, the coding sequence for the mature protein without a signal sequence was amplified with a primer (5′-GATGATGGCCTTACC-3′) which is complementary to the 5′ end of the antigen B cDNA and a primer [5′-$(GA)_6$AT TCCGGGTACCGGGCCCCCCC-3′] which is complementary to the pBluescript vector including an internal *Eco*RI site at the 5′ end. The PCR fragment was cloned into the *Sma*I and *Eco*RI sites of plasmid pGEX-3X. For purification of the glutathione *S*-transferase (GST) fusion protein, the antigens were synthesized in *E. coli* DH5α by induction of gene expression with 10 mM isopropyl-β-D-thiogalactopyranoside for 1 h. After centrifugation at 6,000 × *g*, the cells were lysed by the addition of lysozyme (10 μg/ml) and Triton X-100 (final concentration, 1% [vol/vol]) and were subsequently sonicated. After centrifugation at 15,000 × *g*, the supernatant was incubated with glutathione-Sepharose (Pharmacia Biosystems). The fusion proteins were eluted with 5 mM glutathione, as recommended by the manufacturer.

MABs. Monoclonal antibodies (MAbs) against the purified recombinant antigens EM10 and EG55 were raised by immunization of BALB/c mice with 50 μg of purified GST fusion proteins at weekly intervals. The first immunization was performed with complete Freund's adjuvant; the five following booster injections were performed without Freund's adjuvant. Generation of hybridoma cells was performed by standard protocols (8). Typing of the classes and subclasses of the MAb was performed with a subtyping kit (Boehringer, Mannheim, Germany). MAb 2810, which is directed against EM10, is an immunoglobulin G2a antibody; the EG55-specific MAb 14C11 is of the immunoglobulin G1 class. Ascitic fluids of both MAbs were produced by intraperitoneal injection of 10^5 hybridoma cells into BALB/c mice. Antibodies were purified from the ascitic fluid with protein A-Sepharose as described previously (6).

Sandwich ELISA for serological differentiation between *E. multilocularis* and *E. granulosus* infections. U-formed polystyrene microtiter plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 20 μl of purified MAb 2810 or MAb 14C11, respectively (5 μg of each antibody per ml of phosphate-buffered saline [PBS; pH 7.4]). Wells were saturated for 30 min with 200 μl of 2% (wt/vol) fat-free milk in PBS and were subsequently washed three times with 200 μl of PBS. Twenty microliters of purified GST-EM10 or GST-EG55 (5 μg of each recombinant antigen per ml diluted in PBS) was added, and the mixtures were incubated at room temperature for 90 min. After washing three times with PBS, 20 μl of patient sera, diluted 1:300 in 2% (wt/vol) fat-free milk in PBS, was added and the mixture was left for 1 h at room temperature. Plates were washed again three times with PBS. The presence of antibodies in the sera directed against antigen EM10 or EG55 was detected by the addition of 20 μl of horseradish peroxidase-labeled goat anti-human immunoglobulin (Dianova, Hamburg, Germany), diluted 1:5,000 in 2% (wt/vol) fat-free milk in PBS. After incubation for 1 h at room temperature and washing three times with PBS, 20 μl of substrate solution was added; the substrate solution consisted of 10 mg of 2,2-azino-bis-3-ethylenbenzothiazoline-6-sulfonic-acid (Boehringer) per ml and 0.1% H_2O_2 in 10 mM sodium phosphate buffer (pH 6). Extinction was measured after 30 min at 414 nm in a Titertek Multiscan instrument (Flow Laboratories, Meckenheim, Germany). All sera were tested in duplicate in both assay systems at the same time to avoid variabilities. As a control, all sera were tested in two additional enzyme-linked immunosorbent assay (ELISA) systems which were identical to those described above, with the exception that the recombinant antigens were omitted to exclude false-positive reactions based on interactions of human immunoglobulins with the MAbs, i.e., by rheumatoid factors. The extinction values obtained for the control assays were subtracted from the values for the ELISAs, which were performed with the recombinant antigens. A mean value of 39 for normal sera tested in each ELISA plus 2 standard deviations was taken as the cutoff.

Patient sera. All echinococcal sera were obtained from patients with histologically confirmed *E. multilocularis* or *E. granulosus* infections. Thirteen serum samples (5 from patients with *E. multilocularis* and 8 from patients with *E. granulosus* infections) were obtained from the Department of Abdominal Surgery, Medical School of Hannover. Sixteen serum samples from patients infected with *E. multilocularis* and 1 serum sample from a patient infected with *E. granulosus* were kindly provided by H. Auer, Institute for Hygiene, University of Vienna, Vienna, Austria; 25 serum samples (16 from *E. multilocularis*-infected patients and 9 from *E. granulosus*-infected patients) were from the Medizinische Klinik at the University Ulm, Ulm, Germany; and 75 serum samples (37 from *E. multilocularis*-infected patients and 38 from *E. granulosus*-infected patients) were a generous gift of E. Mannweiler, Bernhard-Nocht-Institut, Hamburg, Germany. An additional eight serum samples from *E. granulosus*-infected patients were kindly provided by K. Janitschke, Robert Koch-Institut, Berlin, Germany. Forty-six serum samples were from *E. granulosus*-infected patients with hepatic localization of the parasite, three serum samples were from patients with pulmonary localized cysts, two patients suffered from cysts in the liver and lung, one patient had cysts in the liver and brain, four patients had cysts in the kidneys, two patients had cysts in the whole peritoneum, and one patient had a cyst in the quadriceps femoris. The organ localization of *E. multilocularis* was the liver (58 patients), liver and lungs (5 patients), liver and kidney (1 patient), and liver and three or more other organs (5 patients). No data were available about the localization of the parasite for five of the patients. All serum samples from *E. multilocularis*- and *E. granulosus*-infected patients tested

exhibited antibodies against total larval antigen when tested in the ELISA and/or hemagglutination assay by using crude *E. multilocularis* metacestode tissue and hydatid cyst antigen (22). Additional serum samples from patients with other parasitic infections were tested as controls; sera from patients with neurocysticercosis (n = 17), filariasis (n = 3), fascioliasis (n = 2), amebiasis (n = 2), schistosomiasis (n = 4), and *Paragonimus* infection (n = 3) were kindly provided by U. Bienzle, Landesinstitut für Tropenmedizin, Berlin, Germany; B. Gottstein, Institute of Parasitology, Bern, Switzerland; and E. Mannweiler, Bernhard-Nocht-Institut, Hamburg, Germany, or were from the Institute for Medical Microbiology, Hannover, Germany. An additional 39 serum samples were from individuals with no clinical signs of a parasitic infection.

RESULTS AND DISCUSSION

Serum specimens from 74 patients with alveolar echinococcosis and serum specimens from 64 patients with *E. granulosus* infections were tested in two separate sandwich ELISAs by using GST fusion proteins of antigen EM10, which we previously described as an *E. multilocularis*-specific antigen (10), and of the 12-kDa subunit of antigen B, termed EG55. Although the antigens were purified by affinity chromatography by using their glutathione moieties, the purified fusion proteins were still contaminated with *E. coli* antigens, causing false-positive reactions because of the *E. coli*-specific antibodies in patient sera (9). However, these false-positive reactivities could be avoided by introduction of a second affinity purification step, i.e., capturing of the fusion proteins via MAbs in microtiter plates. The use of GST fusion proteins for the serodiagnosis of echinococcal disease may lead to false-positive results in patients suffering from schistosomiasis, since 47% of those patients exhibit immunoglobulin G antibodies against GST (2). However, we did not detect anti-GST antibodies in the sera from patients with schistosomiasis used in the present study. Even though cloning in expression vector pGEX-3X enables enzymatic cleavage of the GST moiety by factor X, we were not able to cleave the GST moiety from the GST-EG55 fusion protein, in contrast to the case with the GST-EM10 fusion protein; this was presumably due to steric hindrance of the factor X cleavage site in this construct. However, echinococcosis and schistosomiasis can, in most cases, clearly be distinguished by clinical criteria, thus rendering the serological differentiation between both parasitic infections unnecessary.

To determine the cutoff value of both ELISAs, we tested 39 serum samples from healthy people with no signs of a parasitic infection. The mean plus two standard deviations was taken as the cutoff. As summarized in Fig. 1, we found by this strategy that 69 of 74 serum samples (93.2%) from patients with alveolar echinococcosis were reactive with antigen EM10. Twenty-nine of these 69 EM10-positive serum samples were also reactive with EG55 (39.2%). One of the serum samples from a patient with *E. multilocularis* infection was EM10 negative but reacted with EG55. Four serum samples were negative in both ELISAs. From the 64 serum samples from *E. granulosus*-infected patients, 57 were reactive with EG55 (89.1%). One of the EG55-positive and one of the seven EG55-negative serum samples reacted with antigen EM10. The 31 control serum samples collected from patients with other parasitic infections did not react with EG55. In contrast, from the 17 serum samples from patients with neurocysticercosis, 3 were positive with antigen EM10 (17.6%), but none of the other control sera from

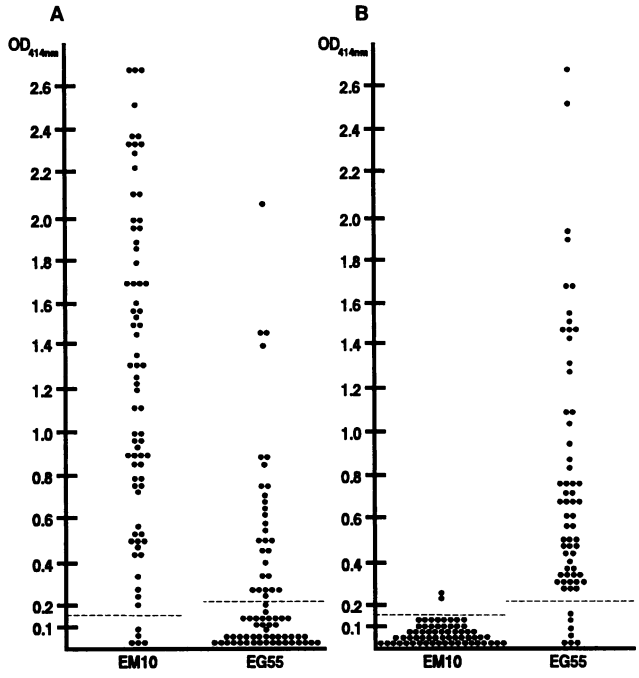

FIG. 1. Extinction values obtained in the EM10 and EG55 ELISAs for 74 serum samples from *E. multilocularis*-infected patients (A) and 64 serum samples from *E. granulosus*-infected patients (B). All sera, including the controls for determination of the cutoff, were tested three to four times. The results of one representative assay are given. A variation of the cutoff value between optical densities at 414 nm (OD_{414}) of 0.14 and 0.2 for the EM10 ELISA and between optical densities at 414 nm of 0.15 and 0.28 for the EG55 ELISA was calculated in the independently performed tests. The variability of the cutoff values coincided with the variability of the values obtained from the patient sera, thus giving consistent positive or negative results for each serum sample tested. The dashed lines indicate the cutoffs.

patients with *Filaria*, *Entamoeba*, *Fasciola*, *Schistosoma*, or *Paragonimus* infections reacted with EM10. All sera were tested three to four times, including the sera used for determination of the cutoff values, to exclude variations from day to day. However, all independently performed ELISAs provided the same results. This high level of reproducibility of both ELISAs is most important when there are low serum antibody titers against EM10 or EG55, which exhibit extinction values slightly above the cutoff.

The specificity of EM10 found in the present study contrasts to our previous findings (10), in which we described a 100% specificity of EM10 for the serodiagnosis of alveolar echinococcosis, including sera from patients with neurocysticercosis infection. However, it should be noted that in the present study more sera from patients with neurocysticercosis infections were tested and the test system applied in the present study was proven to be more sensitive. Despite its limited specificity, EM10 enables a highly specific differentiation between *E. multilocularis* and *E. granulosus* disease. Of 64 serum samples from patients with *E. granulosus* infections, only 2 showed weak reactivities with EM10. These two serum samples exhibited extinction values of 0.23 and 0.24, which were slightly above the cutoff value of 0.15. In contrast, this high species specificity was not found for EG55. Initially, the 12-kDa antigen B subunit was described as an *E. granulosus*-specific antigen (31), but this could not

be confirmed in subsequent studies (23, 24). Maddison et al. (24) observed antibodies in the sera of 39% of all patients infected with *E. multilocularis*, which is exactly the same result that we observed in the present study (39.2%). However, the combined use of the recombinant antigens EM10 and the recombinant low-molecular-weight subunit of antigen B, EG55, enables a highly sensitive and highly specific means of differentiating between *E. multilocularis* and *E. granulosus* disease. When all EM10-positive and all EM10-plus-EG55-positive sera from patients with histologically confirmed alveolar echinococcosis are considered, an *E. multilocularis* infection can be diagnosed with a sensitivity of 93.2%. *E. granulosus* infections can be diagnosed by a serum reactivity against EG55 but negative results in the EM10 ELISA. By using these criteria for the analysis of 64 serum samples from patients with histologically confirmed cystic echinococcosis, a sensitivity of 89.1% was achieved. Considering the false-positive EM10 results for two serum samples from *E. granulosus*-infected patients and a false-positive result with one serum sample from a *E. multilocularis*-infected patient, the specificity for serological differentiation of an *E. multilocularis* infection is 96.8%, and it is 98.6% for an *E. granulosus* infection. The sensitivity of 91.4% for the diagnosis of alveolar echinococcosis with antigen EM10 is similar to the results obtained with the *E. multilocularis* Em2a antigen, which detects 95% of histologically confirmed cases of alveolar echinococcosis (14). However, the specificity of the EM10 antigen is strongly improved compared with those of crude antigens. Furthermore, the sensitivity for the serodiagnosis of *E. granulosus* infections by the EG55 ELISA is similar to that found for other assay systems based on antigen B isolated from hydatid fluids (13, 34). Because the native antigen B of the hydatid fluid may be glycosylated (38), the carbohydrate moiety is not an essential antigenic part for serodiagnostic purposes. However, use of the recombinant subunit of antigen B is attractive in terms of large-scale production and standardization of assay systems.

It should be noted that the EM10 and EG55 ELISAs described here enable serological verification and differentiation between echinococcal infections caused by both organisms, but they do not provide a method for quantification of antibody titers in patient sera, since only one serum dilution of 1:300 is tested. We analyzed those sera which were negative in this serum titration in dilutions of 1:200 and 1:100 as well and, for this purpose, also redetermined the cutoff value by using equal dilutions of control sera from healthy individuals. However, all sera which were negative when used at 1:300 dilutions were also negative when used at lower serum dilutions. Although the sensitivities of the ELISAs are high, they are not sufficient for large-scale seroepidemiological studies in areas in which echinococcoses are of low prevalence. For these reasons, screening for echinococcosis and long-term observations of antibody titers in infected patients should be performed with crude echinococcal antigen preparations in ELISAs or hemagglutination assays, which provide a higher sensitivity than the ELISAs described here (13). Positive sera in these assay systems can then be differentiated by the ELISAs with the recombinant antigens.

The sensitivity of assay systems based on genetically engineered proteins may be improved by inclusion of additional recombinant antigens. However, combination of antigen EM10 with the recently described recombinant antigen EM13, which is specifically expressed in *E. multilocularis* larvae but not in *E. granulosus* larvae (11), did not improve the sensitivity when applied to a sandwich ELISA comparable to those described here (9). Recently, Gottstein and coworkers (25) combined the recombinant antigen II/3-10 with the biochemically purified antigen Em2a, resulting in a sensitivity of 97% and a specificity of 99% (16). However, EM2a is not a recombinant antigen, and therefore, purification by affinity chromatography requires cultivation of *E. multilocularis* metacestodes in laboratory animals. Therefore, definition of additional recombinant antigens is warranted for further improvement of diagnostic assays for echinococcal disease.

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