Close Relationship between Clinical Regression and Specific Serology in the Follow-up of Patients with Alveolar Echinococcosis in Different Clinical Stages

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Abstract. We compared four enzyme-linked immunosorbent assays (ELISAs) with 172 serum samples from 28 patients with alveolar echinococcosis in different clinical stages according to the World Health Organization–PNM (P = parasitic mass in the liver, N = involvement of neighboring organs, M = metastasis) staging system. The sequential antibody responses against Em2plus, Em10, and Em18 antigens, and a crude antigen extract were measured in cohorts with resected and unresected lesions. Antibody levels in all assays correlated with the PNM stage before treatment, and the highest correlation was shown for the Em18 assay. The PNM stage did not influence the antibody kinetics, but changes in antibody levels depended on the treatment. In patients after curative surgery, seroreversion in the Em2plus ELISA indicated successful resection of lesions in more patients than any other assay, irrespective of the clinical stage. There were no significant differences in the time before assays that use recombinant or purified antigens became unre-active. Antibodies directed against crude antigens were detectable longer than other antibodies in all patient cohorts and stages.

INTRODUCTION

Alveolar echinococcosis (AE) is an important parasitic zoonosis of the northern hemisphere. It is caused by the larval stage (metacestode) of the fox tapeworm Echinococcus multilocularis. The disease is characterized by infiltrative growth of the metacestode in the liver of suitable natural intermediate hosts and accidentally in humans. Because metastasis may occur, the biological characteristics of the metacestode are similar to a malignant tumor. Radical resection of parasitic lesions is the preferred treatment, but the timely detection of recurrence after surgery is difficult. However, most patients are inoperable at the time of diagnosis. In such cases, the determination of disease activity (i.e., growth of parasitic lesions) and response to antiparasitic chemotherapy is important. Imaging tools, such as ultrasound and computed tomography are currently the basic monitoring techniques. Enlargement of lesions is slow and demonstration of progressive AE by imaging requires a period of several months. Disease progression can therefore only be evaluated retrospectively by imaging. \(^{1}\) \(^{18}\)F-fluorodeoxyglucose positron-emission tomography (FDG-PET) has also been used to monitor disease activity. However, the results of this real-time investigation are controversial.\(^{2,4–6}\) Serologic analysis with affinity-purified and recombinant antigens has recently shown a promising correlation with disease activity.\(^{7,8}\)

In this study, we tested four enzyme-linked immunosorbent assay (ELISAs) in parallel with serum samples from 28 patients with resected or unresected parasitic lesions in different clinical stages of AE according to the World Health Organization (WHO)–PNM (P = parasitic mass in the liver, N = involvement of neighboring organs, M = metastasis) system.\(^{9}\) The sequential antibody responses against two recombinant antigens (Em10 and Em18), an affinity-purified antigen combined with a recombinant antigen (Em2plus), and a crude antigen extract were measured and compared in 172 serum samples.

PATIENTS AND METHODS

Patients. All patients in this study attended the University Hospital and Medical Center in Ulm, Germany. A total of 28 patients with the history of hepatic AE and a follow-up period of 1.5–6.5 years were included in the study. The patients (age range = 17–74 years, mean age = 51.2 years, sex ratio [M:F] = 0.4:1) were in different clinical WHO–PNM stages of the disease. All patients had acquired AE in Germany and received benzimidazole therapy. Twelve patients had curatively resected lesions, 2 had recurrences after surgery, 1 had a palliative resection only, 11 had unresectable lesions but stable disease, and 2 had apparently dead, fully calcified lesions (Table 1). Serum samples were tested at the Institute of Hygiene and Microbiology, University of Würzburg, Germany (crude larval antigen-, Em2plus- and Em10-ELISA), and at the Department of Parasitology, Asahikawa Medical College, Asahikawa, Japan (Em18-ELISA) in a blind test. Classification of curative resection, stable disease, progressive disease, or presence of an apparently dead, fully calcified lesion was established by magnetic resonance imaging on the basis of lesion size and morphology at the respective follow-up intervals. The study was reviewed and approved by the ethics committee of the University of Ulm.

Methods. For the crude larval antigen ELISA, E. multilocularis metacestode tissue harvested from the peritoneal cavity of Mongolian jirds was mechanically homogenized and centrifuged. The supernatant was used to coat microtiter plates at a concentration of 2 ng/µL. Patient serum samples were tested at a dilution of 1:300 after pre-absorption of the wells with 2% skimmed milk (Merck, Darmstadt, Germany). Serum antibodies bound to echinococcal antigens were detected by secondary peroxidase-conjugated anti-human IgG antibodies (Dako, Glostrup, Denmark) using 2,2′-azino-di-(3-ethylbenz-thiazoline sulfonate) (ABTS) (Roche, Mannheim, Germany) as chromogenic substrate. Absorbance was measured after 60 minutes at 410 nm with a reference wavelength of 490 nm. For
Characteristics of 28 patients with alveolar echinococcosis included in the study

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<th>Patient no.</th>
<th>Stage</th>
<th>PNM* code</th>
<th>Status†</th>
<th>Age, years‡</th>
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* PNM = P = parasitic mass in the liver; N = involvement of neighboring organs; M = metastasis.
† Assessed by imaging (apparently dead lesion, progressive disease and stable disease) or imaging and histologic analysis (curative resection).
‡ Age when first blood sample was obtained.

The Em2plus ELISA (Bordier Affinity Products, Crissier, Switzerland) was used according to the manufacturer’s instructions. Indices for the Em2plus ELISA were calculated by dividing the individual serum sample absorbance by the provided weak positive control serum (cut-off serum). For the Em10 ELISA, microtiter plates were coated with recombinant Em10 antigen at a concentration of 10 ng/µL. Patient serum samples were tested as described for the larval antigen extract ELISA. For the Em18 ELISA, recombinant Em18 antigen was used to coat microtiter plates at a concentration of 100 ng/well. Patient serum samples were tested at a dilution of 1:100 after pre-absorption of the wells with 1% casein in 20 mM Tris-HCl (pH 7.4)-150 mM NaCl buffer. Serum antibodies bound to echinococcal antigens were detected by secondary peroxidase-conjugated protein G (Zymed, South San Francisco, CA) using ABTS (Sigma) as chromogenic substrate. Absorbance was measured after 30 minutes at 405 nm with a reference wavelength of 630 nm. For the calculation of the cut-off, the mean value of the absorbance of 40 sera from healthy blood donors was added to 3 x the SD. The index of the individual serum sample was calculated by dividing the sample’s absorbance by the cut-off.

Statistical analysis. Statistical analyses were done with the free software environment R for statistical computing. Non-parametric data were analyzed using Spearman’s rank test for the correlation of the clinical stage and the ELISA index of the respective assays. Wilcoxon’s rank sum test was used to examine differences in the time to negativity of the various assays. P values < 0.05 were regarded as significant.

RESULTS

The height of the ELISA index of all tests correlated weakly with the clinical PNM stage prior to treatment. The highest correlation was shown for the median values of the Em18 ELISA (Figure 1). The PNM stage per se did not influence the kinetics of the antibodies but changes of antibody levels strongly depended on the type of treatment the individual patient underwent in each stage (Figure 2A and B). In general, there were good agreements in the patterns of all assays tested.

In the cohort of 12 patients who underwent curative resection, antibody levels decreased rapidly after resection of the parasitic mass and the surrounding inflammatory tissue in all assays. Levels of antibodies decreased below the cut-off level of the respective assays (Figures 2A and 3A). In some patients, two or all three ELISAs, which used recombinant and/or purified antigens, became unreactive at the same time. The time elapsed before antibodies became undetectable in the various assays is shown in Figure 4. More patients had a decrease in antibody levels below the cut-off when tested by the Em2plus assay than by the Em10 assay or the Em18 assay (Figures 3A and 4). The time before antibodies became undetectable was significantly shorter in the Em2plus assay than in the crude antigen ELISA (P = 0.013). No significant differences in the time in negativity could be demonstrated between...
the Em\textsuperscript{2plus}, Em10, and Em18 assays. The Em18 index demonstrated the most marked decrease of all assays during the first follow-up interval in all patients and PNM stages in this cohort (Figure 2A).

Once the antibody levels decreased below the cut-off, they remained undetectable throughout the observation period in all assays. The decrease below the cut-off level reflected serologically the clinical regression in this patient cohort as assessed by follow-up imaging. However, the indices of the Em\textsuperscript{2plus}, Em10, Em18, and crude antigen ELISA did not decrease below the cut-off in 2 patients (28 and 25), 3 patients (28, 4, and 24), 5 patients (2, 13, 24, 25, and 27), and 4 patients (7, 24, 25, and 28) at the end of their follow-up periods (19 and 34 months; 19, 34, and 36 months; 30–66 months; and 19–36 months), respectively. In patients who did not serorevert in the various tests, no regrowth of parasitic tissue was detected by imaging during a follow-up period of 1.5–5.5 years. This finding also applies to the three patients in stage IV without seroreversion for three different assays each (patients 24, 25, and 28). There was no test consistently unreactive for these persons. There was no association of the PNM stage and the time to negativity in the different assays. Indices of antibodies against all antigen preparations used showed similar kinetics in this patient cohort, irrespective of the individual PNM stage.

In the three patients after non-curative resection, all antibody levels showed an initial decrease. However, an increase was demonstrated again in stage IV without seroreversion for three different assays each (patients 24, 25, and 28). There was no test consistently unreactive for these persons. There was no association of the PNM stage and the time to negativity in the different assays. Indices of antibodies against all antigen preparations used showed similar kinetics in this patient cohort, irrespective of the individual PNM stage.

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The observation period in all assays (Figures 2B and 3B). No differences in antibody kinetics in this patient cohort in different PNM stages were observed. Antibodies against the crude antigen preparation never decreased below the cut-off. In contrast, antibodies against Em\textsuperscript{2plus}, Em10, and Em18 decreased below the cut-off in 4 patients (16, 6, 8, and 11), 6 patients (10, 15, 8, 16, 6, and 11), and 2 patients (8 and 11) after 8, 18, 51, and 72 months; 6, 11, 15, 21, 21, and 72 months; and 15 and 72 months, respectively. The slight decrease in antibody levels paralleled serologically the clinically stable disease in this patient cohort as assessed by follow-up imaging. The Em18 index demonstrated the most pronounced decrease of all assays during the first follow-up interval in all patients and PNM stages of this cohort (Figure 2B). There were no clinical or radiologic differences between patients who showed seroreversion in assays using recombinant or purified antigens and those who did not serorevert during the follow-up periods.

In the two patients with apparently dead lesions, antibody levels showed a steady decrease. Antibodies against the crude antigen preparation remained above the cut-off for 24 and 50 months, whereas the Em\textsuperscript{2plus}, Em10, and Em18-assays all
showed negative results after 12 and 30 months (patients 17 and 5), respectively.

**DISCUSSION**

Determination of disease activity is of major importance in patients with AE. Possible recurrences should be detected timely after resection of parasitic tissue and progressive disease should be monitored closely in cases with unresectable lesions. Imaging tools enable only a retrospective evaluation of disease activity, which is reflected by growth of the metacestode. Results of real-time FDG-PET investigations are currently debated and the equipment is not readily available in many centers. No exact statement on disease activity can be given without histologic examination of excised tissue.\(^4\) However, many areas of the parasitic lesion would have to be examined because growth of the parasitic tissue occurs focally at the periphery of the lesion(s). Surrogate parameters that have been used to monitor disease activity in AE include serum zinc concentrations,\(^12\) total serum IgG and IgE,\(^12\) and host alkaline phosphatase.\(^5\) Moreover, genus-specific serologic analysis measuring IgG, IgG subclasses, and IgE directed against crude metacestode antigen preparations,\(^7,8,13–18\) and species-specific serologic analysis measuring IgG against recombinant or purified antigens\(^7,8,19–21\) has been used and correlation with disease activity has been demonstrated. However, patients have not been grouped according to their clinical stage.

In this study, we conducted a serologic follow-up of patients with AE grouped according to the WHO-PNM staging system with multiple tests. Four different assays were run in parallel and good agreements in the patterns of all assays were shown. The study demonstrated that the kinetics of antibody levels to *Echinococcus* were not influenced by the PNM stage. However, the height of antibody levels prior to treatment was dependent of the PNM stage. Em18 indices showed the highest, although relative weak, correlations with the PNM stages prior to treatment, a classification that is based on imaging results. As described previously, the highest antibody levels occur among inoperable cases.\(^20\)

Hypergammaglobulinemia is a frequently encountered phenomenon in patients with AE,\(^22\) and IgG (especially IgG4)
Our data of the patient cohort with curatively resected lesions are consistent with previous findings that levels of antibodies against Em2plus, Em18, and crude antigen preparations can decrease below the cut-off. In this study, antibodies were undetectable in more patients after curative resection when measured in the Em2plus ELISA than in the Em10 or Em18 ELISA, irrespective of the individual PNM stage. Many tests showed unreactive results after the same follow-up interval in some patients. None of the assays that use recombinant or purified antigens reversed significantly faster than the other after curative surgery. It is possible, however, that some tests become unreactive earlier than others, when tested in a shorter interval. Our data confirm reports of a more rapid decrease of levels of antibodies against recombinant antigens than levels of antibodies against crude antigen extracts. However, the presence of viable parasitic tissue will be unlikely if the index of a test that uses a crude antigen preparation decreases below the cut-off. Some patients did not serorevert in certain assays although a recurrence during follow-up could not be demonstrated by imaging. Of this patient subgroup, most persons were in an advanced stage of AE (stage IIIa and IV, respectively). The follow-up period may not have been long enough in these patients to demonstrate either seroreversion in all assays or recurrence of disease by imaging.

In patients with recurrences, antibody levels initially decreased but increased again during the follow-up period. Similar results have been observed with the Em2plus ELISA, the Em18 ELISA, and the Em18 Western blot.

In the patient cohort with unresectable lesions, our data also mirror previous results of elevated and slowly decreasing antibody levels in the Em2, Em2plus, Em18, and crude antigen assays. In our subgroup of patients with unresectable lesions and undetectable antibodies against recombinant or purified antigens, a regression of lesions towards death of the parasite might be possible. This was shown by previous analyses for the Em2plus and Em18 assays. In our patients, the follow-up period may not have been long enough to demonstrate either seroreversion in all assays or regression of lesions by imaging.

The kinetics of antibody levels in patients who have apparently dead and fully calcified lesions were similar to the kinetics of the cohort with stable disease and no antibodies against recombinant or purified antigens.

In this study, the median follow-up period of the 28 patients examined was 5 years. A longer observation period of more patients would have been favorable in obtaining a firm basis for the correlation of serologic results and clinical condition. However, AE is a rare disease and therefore not many patients can be included in such studies.

In conclusion, serologic analysis shows a close relationship between clinical status and treatment of patients with AE. The PNM stage does not influence antibody kinetics. The Em18 index mirrors best the clinical PNM stage prior to treatment. It shows the greatest changes in all patient cohorts, clinical stages, and treatments in the early phase of medical intervention. Thus, results of the Em18 assay seem to be the easiest to interpret. The Em2plus ELISA may signal successful resection of parasitic tissue in an early stage of infection.
of lesions in more patients than assays using Em10 or Em18. None of the assays that use recombinant or purified antigens reverses significantly faster than the other after curative surgery. Tests employing crude antigen extracts of the parasite are the safest for detecting remaining parasitic tissue in all clinical stages. However, these tests are not useful in determining disease activity because of the long period that they need to become unreactive. Although serologic analysis is cheaper than imaging techniques, immunological test results should still be interpreted in conjunction with imaging data.

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