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INTRODUCTION

Alveolar echinococcosis (AE) is an important parasitic zoonosis of the northern hemisphere. AE 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. As metastasis formation may occur, the biological characteristics of the metacestode are similar to a malignant tumor. Radical resection of parasitic lesions is the preferred treatment,[1] but the timely detection of recurrence after surgery is difficult. Most patients are inoperable at the time of diagnosis, however.[2,3] 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 (CT) are currently the basic monitoring techniques.[4] 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.[2] [^{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.[2,4,5,6] Serology with affinity-purified and recombinant antigens has recently shown a promising correlation with disease activity.[7,8]

In this study, we tested four different ELISAs in parallel on sera of 28 patients with resected or unresected parasitic lesions in different clinical stages of AE according to the WHO-PNM-system [9] for the first time. The sequential antibody responses against two recombinant antigens (Em10, Em18), an affinity purified antigen combined with a recombinant antigen (Em2^{plus}), and a crude antigen extract were measured and compared in a total of 172 sera.

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PATIENTS AND METHODS

Patients. All patients incorporated in this study attended the University Hospital and Medical Center 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 into the study. The patients (age range 17--74, mean age 51.2, sex ratio [m:f] 0.4:1) were at different clinical WHO-PNM stages of the disease. All patients had acquired AE in Germany and received benzimidazole therapy. 12 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). The serum samples were tested at the Institute of Hygiene and Microbiology, University of Würzburg, Germany (crude larval antigen-, Em2^{plus}- and Em10-ELISA), and at the Department of Parasitology, Asahikawa Medical College, Japan (Em18-ELISA) under a blind test. The classification of curative resection, stable disease, progressive disease or presence of an apparently dead, fully calcified lesion was established by magnetic resonance imaging based on lesion size and morphology at the respective follow-up intervals. The study was 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. Patients' sera were tested at a dilution of 1:300 after pre-absorption of the wells with 2% skimmed milk (Merck, Germany). Serum antibodies bound to echinococcal antigens were detected by secondary peroxidase-conjugated anti-human IgG-antibodies (DAKO, Denmark) using 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate) ([ABTS], Roche, Germany) as chromogenic substrate. Absorbance was measured after 60 min at 410 nm with a reference wavelength of 490 nm. For the calculation of the cut-off, the mean value of the absorbance of

12 sera from healthy blood-donors was added to 3 x SD. The index of the individual serum sample was calculated by dividing the sample's absorbance by the cut-off.

The Em2^{plus}-ELISA (Bordier Affinity Products, Switzerland) was used according to the manufacturer's instructions. Indices for the Em2^{plus}-ELISA were calculated by dividing the individual serum absorbance by the provided weak positive control serum (cut-off serum). For the Em10-ELISA, microtiter plates were coated with recombinant Em10 antigen [10] at a concentration of 10 ng/µl. Patients' sera were tested as described for the larval antigen extract ELISA. For the Em18-ELISA, recombinant Em18 antigen [11] was used to coat microtiter plates at a concentration of 100 ng/well. Patients' sera 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, USA) using ABTS (Sigma, Germany) as chromogenic substrate. Absorbance was measured after 30 min 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 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, 2B). 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. Antibodies could even drop below the cut-off level of the respective assays (Figure 2A, Figure 3A). In some patients 2 or all 3 ELISAs, which employ recombinant and/or purified antigens, became unreactive at the same time. The time elapse before antibodies became undetectable in the various assays is shown in Figure 4. More patients had a drop of antibody levels below the cut-off when tested by the $Em2^{plus}$-assay than by the Em10-assay or the Em18-assay, respectively (Figure 3A, Figure 4). The time before antibodies became undetectable was significantly shorter in the Em^{2plus}-assay than in the crude antigen-ELISA ($P = 0.013$). No significant differences in the time to negativity could be demonstrated between the Em^{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 dropped below the cut-off they remained undetectable throughout the observation period in all assays. The drop 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 $Em2^{plus}$-, Em10-, Em18-, and crude antigen-ELISA did not fall below the cut-off in 2 patients (#28, 25), 3 patients (#28, 4, 24), 5 patients (#2, 13, 24, 25, 27), and 4 patients (#7, 24, 25, 28) at the end of their follow-up periods (19, 34 months; 19, 34, 36 months; 30--66 months, and 19--36 months), respectively.

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In patients who did not sero-revert in the various tests, no re-growth of parasitic tissue was detected by imaging during a follow-up period of 1.5--5.5 years. This applies also for the 3 patients in stage IV without sero-reversion in 3 different assays each (patients #24, 25, 28). There was no test consistently unreactive in these individuals. There was no association of the PNM stage and the time to negativity in the different assays. Indices of antibodies against all antigen preparations employed showed similar kinetics in this patient cohort, irrespective of the individual PNM stage.

In the 3 patients after non-curative resection, all antibody levels showed an initial decrease. However, an increase was demonstrated again in all assays after 21, 56, and 60 months, respectively (patients #14, 18, 21). The crude antigen-ELISA and the Em18-ELISA never showed negative results, whereas both the indices of the Em2^{plus}- and the Em10-ELISA fell below the cut-off in the patient with palliative resection (#18) after 50 months (data not shown).

In the cohort of 11 patients with unresectable lesions and stable disease under benzimidazole therapy, antibody levels showed only a slow but steady decrease during the observation period in all assays (Figure 2B, 3B). No differences in antibody kinetics in this patient cohort in different PNM stages were observed. Antibodies against the crude antigen preparation never fell below the cut-off. In contrast, antibodies against Em2^{plus}, Em10, and Em18 dropped below the cut-off in 4 patients (#16, 6, 8, 11), 6 patients (#10, 15, 8, 16, 6, 11), and 2 patients (# 8, 11) after some time (8, 18, 51, 72 months; 6, 11, 15, 21, 21, 72 months, and 15, 72 months), respectively. The slight decrease of the 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 radiological differences between patients who demonstrated a sero-

reversion in assays employing recombinant or purified antigens and those who did not sero-revert during the follow-up periods.

In the 2 patients with apparently dead lesions, antibody levels showed a steady decline. Antibodies against the crude antigen preparation remained above the cut-off for 24 and 50 months, whereas the $Em2^{plus}$, Em10- and Em18-assays all became negative after 12 and 30 months (patients #17, 5), respectively (data not shown).

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 allow 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 a histological examination of excised tissue.[4] However, many areas of the parasitic lesion would have to be examined, as growth of the parasitic tissue occurs focally at the periphery of the lesion(s). Surrogate parameters which 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 serology measuring IgG, IgG subclasses, and IgE directed against crude metacestode antigen preparations [13,14,15,16,17,18], and species-specific serology measuring IgG against recombinant or purified antigens [7,8,19,20,21] has been employed and correlation with disease activity has been demonstrated. However, patients have not been grouped according to their clinical stage before.

In this study, we performed for the first time a serological follow-up of patients with AE grouped according to the WHO-PNM staging system with multiple tests. Four different

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assays were run in parallel and good agreements in the patterns of all assays were shown. The study demonstrated that the kinetics of anti-*Echinococcus*-antibody levels 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, though relative weak, correlations with the PNM stages prior to treatment, a classification which 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) and IgE are significantly increased. [12,15,16,17,18] However, the factors which determine the height of the antibody levels remain to be clarified. The activation level of the individual patient's unprotective Th2-response towards an echinococcal infection as seen in progressive cases [23] might be reflected by the amount of antibodies produced. Moreover, the duration of infection, the size and status of the lesions (i.e. metabolically active and/or growing parasitic mass), as well as the severity of the surrounding inflammation might all contribute to the height of the antibody levels. Parasitic tissue is in direct physical contact with host lymphoid cells [24] and an extensive accumulation of histiocytes and lymphocytes is visible in the active zone in human AE.[25] The capsule of the liver adjacent to AE lesions is often involved in the inflammatory process in form of a localized perihepatitis ("frosted liver").[26] The surrounding host inflammatory zone, which is removed by curative surgery, may thus play a key role in mounting and maintaining antibody levels. Accordingly, patients with cured AE exhibit relative low humoral responses to parasitic antigen.[20]

Our study confirms previous reports that the Em18- and $Em2^{plus}$-assays can be used for the follow-up of patients with AE. In direct comparison, antibodies against Em18 demonstrated the greatest dynamic changeability in all patients, cohorts and PNM stages, irrespective of the individual treatment. Anti-Em10 showed similar kinetics to antibodies directed against Em^{2plus} and the Em10-assay seems thus suitable for the serological follow-up

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of patients with AE. Results comparable to those of our crude antigen-ELISA have been demonstrated for the indirect hemagglutination test which uses a hydatid fluid antigen-extract in early investigations.[13] The crude antigen extract-ELISA is therefore also useful for the serological follow-up of patients with AE. However, as antibodies measured by such crude antigen tests reflect much of the antigenetic spectrum of the parasite, the index changes more slowly than the indices of tests which employ single recombinant or affinity-purified antigens.

Our data of the patient cohort with curatively resected lesions are consistent with previous findings that antibodies against Em2^{plus} [7], Em18 [8] and crude antigen preparations [13] can drop below the cut-off. In this study, antibodies were undetectable in more patients after curative resection when measured in the Em2^{plus}-ELISA than in the Em10- or Em18-ELISA, irrespective of the individual PNM stage. Many tests became unreactive after the same follow-up interval in some patients. None of the assays which 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 antibodies against recombinant antigens than antibodies against crude antigen extracts.[27] However, the presence of viable parasitic tissue will be very unlikely if the index of a test which uses a crude antigen preparation falls below the cut-off. Some patients did not sero-revert in certain assays although a recurrence during follow-up could not be demonstrated by imaging. Of this patient subgroup, most individuals 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 sero-reversion in all assays or recurrence of disease by imaging.

In patients with recurrences, antibody levels initially declined but increased again during the follow-up period. Similar results have been observed previously with the Em2^{plus}-ELISA [21], the Em18-ELISA [8], and the Em18-Western blot.[21]

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In the patient cohort with unresectable lesions, our data also mirror previous results of elevated and slowly decreasing antibody levels in the Em2-[27,28], $Em2^{plus}$-[7], Em18-[8] and crude antigen-assays.[13] 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 $Em2^{plus}$- and Em18-assays.[7,21] In our patients, the follow-up period may not have been long enough to demonstrate either sero-reversion 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 very similar to the cohort with stable disease and negative 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 favourable in order to obtain a firm basis for the correlation of serological results and the clinical condition. However, AE is a rare disease and therefore not many patients can be included in such studies.

In conclusion, serology shows a close relationship between the clinical status and the treatment of patients with AE. The PNM stage does not influence the 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. Results of the Em18-assay thus seem to be the easiest to interpret. The $Em2^{plus}$-ELISA may signal successful resection of lesions in more patients than assays employing Em10 or Em18. None of the assays which 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 very useful to determine disease activity due to the long

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FIGURE LEGENDS

Figure 1

Correlation of PNM stage and ELISA-index prior to treatment. Median index values of the respective assays per PNM stage are displayed. An increase in the median values of the indices in parallel with the clinical stage is visible for all antigen preparations. The $Em2^{plus}$- and Em10-ELISAs demonstrated the lowest median indices in all stages. The Em18-ELISA had the highest indices, except for stage I. No prominent changes of the $Em2^{plus}$-, Em10- and Em18-indices are visible between stages IIIa and IIIb. Between stages IIIb and IV, only the Em18-index shows a marked increase. Spearman's rank correlation coefficients (ρ) for the Em^{2plus}, Em10, Em18, and crude antigen assay and the PNM stages were 0.421, 0.430, 0.483, and 0.319, respectively.

Figure 2

A, Antibody profiles in patients after curative resection. Median index values of all assays from 12 patients in different clinical stages are presented. A rapid decline of all antibody indices is clearly visible. **B,** Antibody profiles in patients with stable disease and unresectable lesions. Median index values of all assays from 11 patients in different clinical stages are presented. A slow decline of all antibody indices is visible.

Serological data were accumulated for intervals of 6 months. At each given interval, data from 2 to 12 patients and 2 to 11 patients were available, respectively. Cut-off level is 1. Time of resection and start of chemotherapy is at month 0, respectively.

Figure 3

A, Antibody indices of all assays from 12 patients in different clinical stages after curative resection. **B,** Antibody indices of all assays from 11 patients in different clinical stages with stable disease and unresectable lesions.

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Serological data were accumulated for intervals of 6 months. At each given interval, data from 2 to 12 patients and 2 to 11 patients were available, respectively. Cut-off level is 1. Time of resection and start of chemotherapy is at month 0, respectively. Horizontal bars represent median values. High initial indices (> 14) of the Em18-assay from patients #13, 25, and 27 are not displayed in Figure 3A.Very high initial indices and follow-up indices (> 15) of the Em18-assay from patients #20, 22, and 26 are not displayed in Figure 3B.

Figure 4

Time elapse before antibodies measured in the $Em2^{plus}$-, Em10-, Em18- and crude antigen-assays fell below the cut-off in patients who underwent curative resection of echinococcal lesions. The PNM stages are symbolized for each assay and patient identification numbers are displayed. Antibodies measured in the $Em2^{plus}$-ELISA fell below the cut-off in 10 out of 12 patients after 5--22 months. Antibodies detected by the Em10-ELISA dropped below the cut-off in 9 patients after 5--24 months and antibodies measured in the Em18-ELISA fell below the cut-off in 7 patients after after 5--22 months. Antibodies tested in the crude antigen-ELISA fell below the cut-off in 8 patients after 16--60 months and remained above the cut-off longer than in any other assay. Significant differences in the time to negativity could only be measured between the Em^{2plus}-assay and the crude antigen-ELISA. Data of patient #13 (stage IIIa) are not shown. In this patient, the index of the crude antigen-ELISA fell below the cut-off after 60 months.

Figure 1