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# Antibodies against *Echinococcus multilocularis* alkaline phosphatase as markers for the specific diagnosis and the serological monitoring of Alveolar echinococcosis

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## SUMMARY

*The immunological properties of the purified alkaline phosphatase (pAP) of Echinococcus multilocularis metacestodes have been investigated using alveolar echinococcosis (AE) patient sera in ELISA tests. A comparative study was done with EmC-Ag (crude antigen) and pAP-Ag (purified antigen). When the parasite purified enzyme pAP was used as antigen, the specificity of the ELISA was markedly increased since it reached 100% without any decrease of its sensitivity (100%). The serologic follow-up of AE patients was conducted during several months with these two antigens in three categories of patients: cured, stabilized and aggravated. There was a good correlation between clinical and serologic data when the pAP was used as antigen in ELISA tests. The anti-pAP antibodies titres did change more rapidly than anti-EmC antibodies titres when a recurrence occurred. Modifications of the anti-pAP antibodies levels were also observed during the patient's therapy: mebendazole, albendazole and Isoprinosine®. These results suggest that pAP-Ag should be used for the diagnosis and the follow-up of AE patients.*

**Keywords** Alveolar echinococcosis, alkaline phosphatase antibodies, specific diagnosis

## INTRODUCTION

The diagnosis of alveolar echinococcosis (AE), a disease caused by the larval stage of the cestode *Echinococcus multilocularis*, can be confirmed by serological techniques using specific antigens such as Em<sup>2Plus</sup> ELISA (Gottstein *et al.* 1993), Western blotting (Ito *et al.* 1993) and by radiological procedures such as ultrasonography and/or CT Scan (Miguet *et al.* 1990). However, the medical follow-up of patients still remains a crucial problem because no method allows clinicians to monitor the alterations or the death of the parasite after the surgical treatment and during chemotherapy. The membrane-bound alkaline phosphatase (pAP) (EC 3.1.3.1.) was chosen as a marker, because of its localization at the host-parasite interface and its very high activity in the *E. multilocularis* metacestodes (Sarciron *et al.* 1991).

In this study, we analysed the diagnosis characteristics of an ELISA developed using pAP as antigen in 37 AE patients and its value as a follow-up marker in 22 AE patients receiving different types of treatment and we show that use of this enzyme as antigen improves the serological diagnosis and monitoring of AE patients.

## MATERIALS AND METHODS

### Antigens

The *E. multilocularis* crude antigen (EmC-Ag) was prepared according to the method described by Vuitton *et al.* 1988. The purified alkaline phosphatase (pAP-Ag) was prepared as follows: all procedures were carried out at +4°C. The *E. multilocularis* metacestodes were homogenized in 100 mM Tris-HCl pH 7.6, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.02 mM ZnCl<sub>2</sub> using a scissor homogenizer, a grinder and then sonicated. The homogenate was

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extracted with n-butanol. This butanolic extract was the first step of the enzyme purification (BE-Ag). The purified *E. multilocularis* alkaline phosphatase was obtained by Concanavalin A-Sepharose chromatography as reported previously by Sarciron *et al.* 1991. The protein content of the antigens was assessed according to Bensadoun & Weinstein (1976).

## Sera

The sera used for the determination of normal ranges and working parameters for the different types of antigens were obtained from 37 healthy donors and 37 AE patients, 15 of them without treatment.

The serum samples used to assess potential nonspecific reactions due to other parasitic or hepatic diseases were obtained from patients (number of patients in brackets) with liver amoebiasis (five), schistosomiasis (11), malaria (three), trichinosis (eight), toxocarosis (seven) and cirrhosis (17). Forty-four sera from patients with cystic echinococcosis (CE) were used to assess the cross-reactivity between the two cestodes.

The patients sera used for monitoring (seven men and 15 women) were taken during a several years medical follow-up (extremes : 35 and 90 months).

These patients were divided into three groups according to the treatment efficacy assessed by clinical and morphological parameters (Wen *et al.* 1995) as follows: cured for nine patients, stabilized for seven patients, aggravated for six patients. AE patients were considered as cured, if clinical symptoms and signs disappeared, if the AE lesion became fibrotic, solid and calcified, or was shown to disappear on ultrasound or CT scan; stabilized, if clinical manifestations were unchanged or complications controlled, and if the AE lesions were morphologically unchanged during the period of treatment; aggravated, if clinical manifestations were aggravated, or the size of the parasite mass increased or the lesion became disseminated, or if recurrence occurred after so-called 'radical' hepatic resection or transplantation (Wen *et al.* 1995). All patients were from Franche-Comté, France. The diagnosis of AE was confirmed by histopathological examination. All sera were tested with EmC-Ag and pAP-Ag enzyme linked immuno-sorbent assay (ELISA).

## ELISA

The ELISA method described by Gottstein *et al.* (1983) was used. The three different antigens were put at the concentration of 5 µg/ml under a volume of 50 µl/well and the patients sera were diluted 1/100. The peroxidase-conjugated goat anti-human IgG (Sigma Chimie, L'Isle d'Abeau, France) was diluted 1/4500.

The optical densities (OD) values were read at 420 nm. All assays were performed at least in triplicate on M 129 B microplates from Dynatech (Flow Laboratories, Paris, France). The results for sequential sera were expressed by the ratio between the patient's OD and the threshold values according to the results of the statistical analysis so that curves of serological values could be easily compared, using EmC and pAP antigens respectively (0.6 for EmC-Ag and 0.12 for pAP-Ag).

## SDS-page

The EmC-Ag (protein content: 9 mg/ml), the BE-Ag (2.6 mg/ml) and the pAP-Ag (700 µg/ml) were solubilized by boiling in the presence of 0.1% SDS and β-mercapto-ethanol for five min. Samples (600 µl) were applied to 5–20% polyacrylamide gels. Standard molecular weight markers were purchased from Sigma (L'Isle d'Abeau, France).

## Electrophoretic transfer

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes (Pharmacia Biotech, Saint-Quentin/Yvelines, France) according to Towbin *et al.* (1979). Protein transfer was carried out using the Pharmacia Multiphor II Novablot semi-dry electrophoresis transfer apparatus at 1 mA/cm<sup>2</sup> for four h.

## Immunochemical staining

Nitrocellulose membrane strips (0.3 cm wide) were blocked with 5% skimmed milk in phosphate-buffered saline (PBS) and then incubated overnight with the patients sera diluted 1/50 with 5% skimmed milk in PBS. The strips were then washed with PBS containing 0.5% Tween-20. Each strip was incubated for three h with peroxidase-conjugated goat anti-human IgG diluted 1/500. The strips were then washed with PBS-Tween followed by PBS for 30 min and soaked in the substrate solution, consisting of 4-chloro-1-naphthol in PBS and the colour development was stopped by distilled water.

## Statistical analysis

Results were expressed as mean and standard deviation of the optical densities obtained using the different *E. multilocularis* antigens under study. Receiver-operating characteristic (ROC) curves (Somoza *et al.* 1989) were used to analyse the diagnostic values of the ELISA using *E. multilocularis* crude antigen and purified alkaline phosphatase antigen respectively.

In the groups of patients with AE (15 patients never treated, on one hand, and 37 patients without any resection

of the lesions but including 22 patients with benzimidazole treatments on the other hand), and in the groups of healthy subjects, patients with CE and patients with other parasitic or hepatic diseases, ROC curves were constructed by plotting the true positive rate (sensitivity) against the false positive rate (1-specificity) of anti-EmC and anti-pAP antibodies at different levels. For both markers it was possible to determine their optimal threshold of discrimination corresponding to the optimal numbers of true positive and false positive patients.

## RESULTS

The first part of this work was devoted to show the immunologic recognition of alkaline phosphatase. After SDS-PAGE and Western blotting of the 55 kDa alkaline phosphatase monomer with AE patients sera, a specific labelling was evidenced (Figure 1).

An enzyme-linked immunoassay (ELISA) with the two different *E. multilocularis* metacestode antigens EmC-Ag

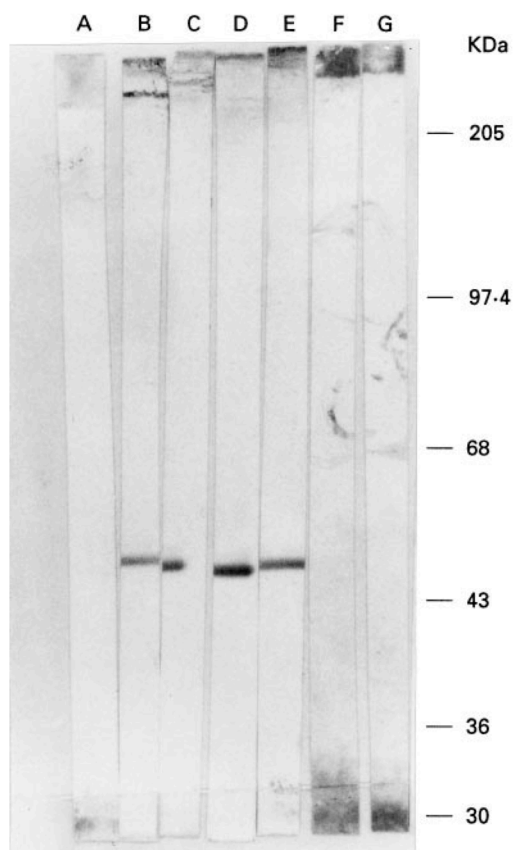
and pAP-Ag was performed. The 37 control sera gave the following OD values,  $0.55 \pm 0.03$  and  $0.05 \pm 0.01$  respectively. The OD values obtained with the 15 sera from untreated AE were:  $1.80 \pm 0.05$  and  $0.48 \pm 0.03$ , respectively. There were highly significant differences between the values obtained with pAP in AE patients and in all the other groups of patients or healthy subjects under study respectively (Figure 2). The results obtained with BE-Ag were intermediary values between those obtained using EmC-Ag and pAP-Ag (data now shown).

Analysis of the ROC curves indicated that for EmC-ELISA the optimal OD values of discrimination in distinguishing between AE patients without treatment and healthy controls ranged between 0.8 and 1.6; for the OD values comprised between 0.8 and 1.6, 100% of AE patients and 100% of controls were classified correctly. The optimal OD values of discrimination in distinguishing between AE and CE patients were 1.6 to 1.8; for these threshold values, sensitivity was 1.0 and 0.93, and specificity was 0.82 and 0.91 respectively.

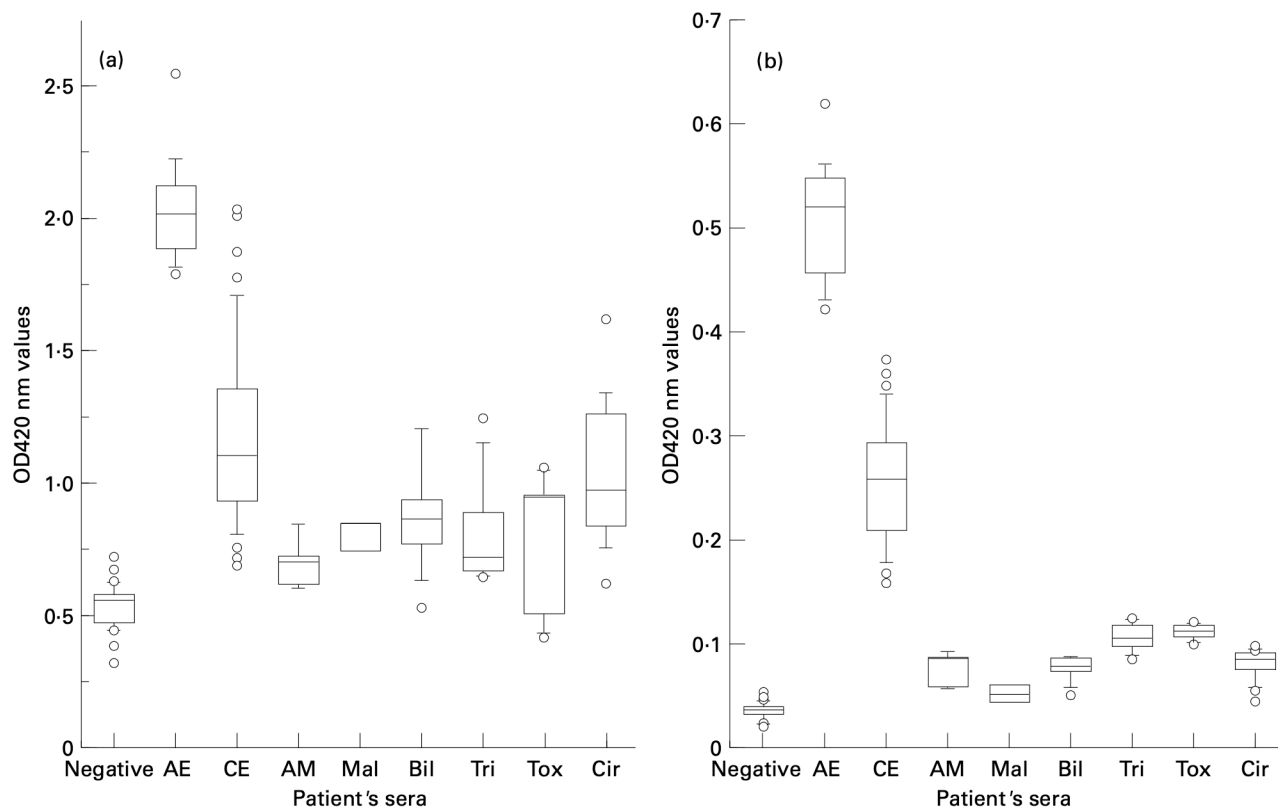
The results were similar when the calculation was made from the values obtained in the series of 37 AE patients. The optimal OD values of discrimination in distinguishing between CE patients and healthy controls were 0.6 to 0.7; for these threshold values, sensitivity was 1 and 0.95, and specificity was 0.84 and 0.97, respectively. For pAP-ELISA, the optimal OD values of discrimination in distinguishing between AE patients without treatment and healthy controls ranged between 0.06 and 0.4 (sensitivity and specificity 100%); the optimal OD values of discrimination in distinguishing between AE and CE patients was 0.04 (sensitivity and specificity 100%). The optimal OD values of discrimination in distinguishing between CE patients and healthy controls ranged between 0.06 and 0.14 (sensitivity and specificity 100%).

Taking into account the results obtained from the different comparisons, using both antigens, it was possible to choose the optimal threshold values for the specific diagnosis of alveolar echinococcosis at OD values of 1.6 and 0.4 for EmC-ELISA and pAP-ELISA respectively. Threshold values for the diagnosis of echinococcosis, irrespective of the species, could be proposed at OD values of 0.6 and 0.12 for EmC-ELISA and pAP-ELISA respectively. These threshold values were thus used to express the antibody levels for the patient follow-ups. results were expressed as the ratio of OD values/threshold values in order to allow comparisons between the course of the antibody levels in a given patient; any value lower than one could thus be considered as a negative result.

The patients under study were grouped according to the parasitologic and therapeutic particularities. Figure 3a-b shows the follow-up of Rac and Mor cured patients. In these



**Figure 1** Recognition of the purified *E. multilocularis* alkaline phosphatase by AE patients sera after Western blotting. Lanes A-F: normal sera; Lanes B-E: AE patients sera. The apparent molecular weights are indicated.



**Figure 2** Results of the ELISA tests using the EmC-Ag (a) and the pAP-Ag (b). The following sera were assayed (number in brackets): controls: negative sera (37); AE: untreated alveolar echinococcosis patients (37); CE cystic echinococcosis (44); Am: liver amoebiasis (5); Mal: malaria (3); Bil: schistosomosis (11); Tri: trichinosis (8); Tox: toxocarosis (7); Cir: liver cirrhosis (18).

two representative patients, one had a partial hepatectomy associated with albendazole while the second patient had been transplanted. A parallel decrease of both anti-EmC and anti-pAP antibodies was observed. It can, however, be noted that the antibody levels did not always reach the threshold values ( $< 1$ , according to the chosen expression of the results), especially when measured using pAP-ELISA.

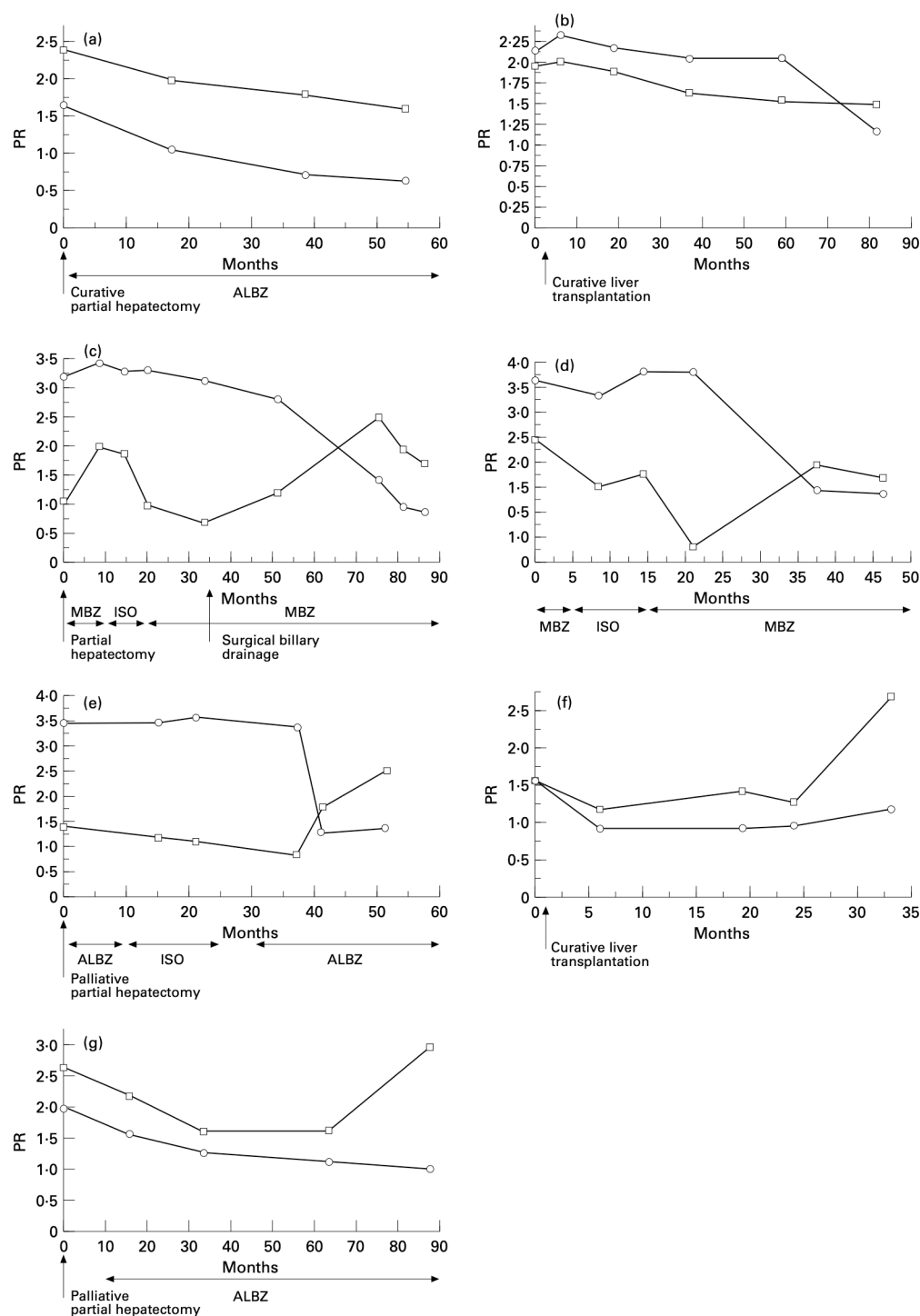
A similar course was observed for the other seven patients of this group. Figure 3c–d represents the follow-up of Pic and Piq; in these and other patients whose clinical evolution was defined as stabilized, we observed a stabilization of both anti-EmC and anti-pAP antibody levels. In patient Pic, surgical and US-guided biliary drainages were associated with a transient increase of anti-pAP antibodies while there was no change (or even a steady decrease) in anti-EmC antibodies (Figure 3c).

Figure 3e,f,g represents the follow-up patients Rec, Vig and Rol whose clinical status was aggravated. A major increase in anti-pAP antibodies was observed whereas anti-EmC antibodies did not undergo very significant variations.

The serologic follow-up of patients who had undergone several drug therapies as for instance Pic, Piq or Rol, showed more important variation in the anti-alkaline phosphatase antibody titres than in the titres of the anti-EmC. Moreover in the stabilized patients we consistently observed an initial increase of anti-pAP antibodies after the beginning of a benzimidazole treatment, and a marked decrease of these antibodies within a few weeks of medical treatment by Isoprinosine<sup>®</sup>. It can be emphasized that both patients, Pic and Piq (Figure 3c–d) had evidence of regression of, or changes in, their liver parasitic mass after Isoprinosine<sup>®</sup> treatment.

## DISCUSSION

The purpose of this study was to evidence the immunogenicity of the *E. multilocularis* metacestode alkaline phosphatase, a property already reported for the *Schistosoma mansoni* enzyme (Pujol & Cesari 1990, Lien *et al.* 1992) and to look for specific variations in the antibody levels induced by surgery and/or chemotherapy. As a matter of



**Figure 3** Serologic monitoring of AE patients by ELISA. The pathological ratio (PR) is the ratio between the OD measured on patient's sera and the threshold discrimination between echinococcosis patients and healthy control patients for the EmC-Ag and for the pAP-Ag. A, b: clinically cured patients, Rac (a) and Mor (b), 53 and 52-year-old females respectively with AE localized in the liver. Rac had undergone a partial hepatectomy in 1991 followed with albendazole treatment, Mor had been transplanted in 1986. C, d: clinically stabilized patients, Pic (c) and Piq (d), 72 and 65-year-old females respectively. Pic. had a hepatic AE and had had a partial hepatectomy in 1987, before medical treatment; hepatic AE lesions invaded the vena cava for Piq who had only undergone several successive medical treatments. E, f, g: clinically aggravated patients, Vig (f) and Rol (g), 43- and 55-year-old males respectively and Rec (e), a 61-year-old female. These three patients had a liver localization. Rec and Rol had undergone palliative partial hepatectomy in 1984 and 1986 respectively, followed, by various treatments. Vig (f) had undergone a transplantation in 1988, although the transplantation was first considered 'curative', it was followed by a recurrence in the graft. The surgical and other therapeutic data are indicated. Tr: liver transplantation; MBZ: mebendazole; ALBZ: albendazole. ○ EmC-Ag; □ pAP-Ag.

fact, it was important to know if the *E. multilocularis* alkaline phosphatase could reflect the parasite viability and therefore the clinical evolution of the patients, since currently no method is able to give us this kind of data, especially when the parasitic burden is very low.

When the parasite purified enzyme pAP was used as antigen, the specificity of the ELISA test was markedly increased, since it reached 100%, without any decrease of its sensitivity (100%, with a series of patients including a very large range of parasite burden, either treated with benzimidazoles or not). This is very interesting for diagnosis, and moreover for mass screenings of echinococcosis, in countries where both AE and CE patients are present, like in China, for example (Craig *et al.* 1992, Wen *et al.* 1992, Ito *et al.* 1993). In this situation, the differential diagnosis is important for both therapeutic indications (markedly different for CE and AE) and epidemiological studies.

Usually, however, there is a relative balance between the sensitivity and the specificity of a diagnostic marker, and it has been clearly shown that anti-Em<sub>2</sub> antibodies, for instance, despite their high specificity were poorly sensitive markers of AE, both for diagnostic use (Gottstein *et al.* 1991) and for mass screenings (Bresson-Hadni *et al.* 1994). For anti-pAP antibodies, ROC curves unequivocally delineated a threshold value between the measurements obtained from AE and CE patients respectively; this strongly suggests a potential use for mass screenings in countries where *E. multilocularis* and *E. granulosus* are sympatric. Preliminary data obtained from 1200 subjects in Xinjiang province (PR China) confirm this suggestion (data not shown). Values obtained in CE patients, nevertheless, were higher than those obtained with control sera, or sera obtained from patients with other diseases. This could indicate a low level of cross-reactivity at the immunological level which, however, does not preclude the use of anti-pAP antibodies for differential diagnosis purpose. The *E. multilocularis* alkaline phosphatase seems to be specific to this parasite, as we reported in a previous study (Sarciron *et al.* 1991b). Although this enzyme shares some biochemical properties with the *E. granulosus* alkaline phosphatase (Lawton *et al.* 1994), it differs by others and a relatively low number of shared epitopes can explain the low level of cross-reactivity with CE sera. On the other hand, the laminated layer localization of the alkaline phosphatase activity (Arsac *et al.* in press), at the host/parasite interface can also lead to a better antigen recognition by the host's lymphocytes since, in AE, there is a very close contact between the larva and the local cellular immune response that does not exist in CE (Vuitton *et al.* 1989, Bresson-Hadni *et al.* 1990).

This high immunogenicity can also explain why anti-pAP antibodies levels were very sensitive to any intervention into

the lesions (surgical operation, drainage), and decreased, albeit steadily, relatively slowly to reach real 'control' levels after complete removal of the lesions. There was a good correlation between clinical and serologic data when the pAP was used as antigen in the ELISA tests for the follow-up of the patients. This was expected if we consider that the metacestode membranous network is the first target hit by chemotherapy and also a key structure in the exchange of nutriment, in which alkaline phosphatase is thought to play an important part (Arme & Pappas 1983, Pappas & Leiby 1986).

It should be noted that some sera from patients who had been initially considered as cured had an increase in their anti-pAP antibody titres that was predictive of recurrence. The case of patient Vig emphasizes the predictive interest of anti-pAP antibodies: the variations of anti-pAP antibodies were particularly noticeable in this patient who had been initially considered as cured and in whom the increase of anti-pAP antibodies preceded the discovery of a parasitic recurrence (using US and CT), although anti-EmC antibodies levels did not change significantly and averaged the threshold value. In the same way, a significant increase in the liver parasitic mass was disclosed in patient Rec, using CT and magnetic resonance imaging. An increase in anti-pAP antibodies could be an indication for a more careful follow-up of patients. Bresson-Hadni *et al.* (1992) have shown that after transplantation, no change in anti-Em<sub>2</sub> IgG antibodies could be detected during the first 2 years of follow-up in patients who had a recurrence and suggested that antibodies against less specific antigens as crude *E. granulosus* antigenic extract, had to be monitored, to distinguish between palliative and curative transplantations. The follow-up of anti-pAP antibodies should thus be added to the current serological monitoring of such patients.

Differential responses of anti-pAP antibodies were noted to two different types of chemotherapy, namely benzimidazoles and Isoprinosine®.

Albendazole and mebendazole have been shown to be parasitostatic and only partially parasitolytic in AE. Their target is however, closely and uniquely, the parasite larval cells (Eckert 1986). The increase in anti-pAP antibodies seen at the initiation or re-initiation of an albendazole or mebendazole treatment could be due to an increased release of the parasitic alkaline phosphatase that secondarily tends toward a stabilization. The mechanism of action of Isoprinosine® is more ambiguous. This drug is an immunostimulant, known to increase cellular immune responses. It has been proposed for the treatment of AE on the basis of experimental studies (Sarciron *et al.* 1991a, 1992). It has been shown that it interferes with the parasite metabolism and growth; however, an effect through an improvement of the host cellular immune responses and/or a shift from TH2 toward TH1 T cell-subpopulations cannot be ruled out.

In fact, we observed a significant increase in the non-specific as well as specific lymphocyte proliferation and a decrease in IgE-dependent immune responses in those patients, treated with Isoprinosine®, who had a significant regression of their parasitic lesions (like patient Pic) or modifications of the CT- and MR-characteristics of the lesions (like patient Piq) (Meyer *et al.* 1991). These observations, together with the decrease of anti-pAP antibodies, could favour an immunologically mediated mechanism of action of Isoprinosine® in AE. The association of increased specific lymphocyte proliferation and decreased antibody (anti-Em<sub>2</sub>) responses in patients with a favourable course of the disease has been shown in another series of patients with AE (Gottstein *et al.* 1991).

The Em<sub>2</sub> antigen is located predominantly in the laminated layer (Hemphill & Gottstein 1995), and has been shown to persist antigenically, independently of the viability status of the parasite and therefore maintain a permanent stimulation of humoral immune response until complete removal of the parasite. This is apparently not the case with the anti-alkaline phosphatase antibodies whose titre seems to follow more closely what happens to the larva. Ito *et al.* (1995) showed a good correlation between the antibody response against Em18 and the presence of active lesions.

It would be interesting to compare the rapidity of apparition of anti-pAP antibodies and the anti-EM18 antibodies titres during the recurrence of a lesion. However, in humans, it will be difficult to correlate the pAP levels to the death of metacystode which can never be ascertained accurately, especially after long-term benzimidazole treatment (Liance *et al.* 1990).

This study, thus, demonstrates the high immunogenicity of the alkaline phosphate of the *E.multilocularis* metacystode in inducing humoral immune response in AE patients. In using this purified enzyme, we increased both sensitivity and specificity of the ELISA tests. On the other hand, with the serologic follow-up of the patients, we were able to show a better correlation between the clinical events and the anti-pAP antibody titres than the anti-EmC antibody titres. The antibody titres did change more rapidly when a recurrence occurred, even in transplanted patients receiving immunosuppression. Treatment with mebendazole seemed to trigger an initial increase of anti-pAP antibodies while albendazole and Isoprinosine® lead to a decrease of these antibodies. This point must be confirmed in a larger number of patients.

Altogether, our results suggest that this new antigen can be proposed to develop standardized ELISA for the diagnosis and the follow-up of AE patients. Its use for mass screenings, which appears very promising, is under current investigation.

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