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RESEARCH BRIEF

Echinococcus multilocularis Metacestodes: Immunological and Immunocytochemical Analysis of the Relationships between Alkaline Phosphatase and the Em2 Antigen

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Echinococcus multilocularis metacestodes possess an alkaline phosphatase (EmAP) which has been extensively characterized at the biochemical level in previous studies. The apparent molecular weight of the enzyme monomer and its isoelectric point matched those originally described for the Em2 antigen, a reference antigen currently used for the immunodiagnosis of *E. multilocularis* infection. These observations raised questions about the molecular relationship between the two molecules. In order to investigate the relations between EmAP and the Em2 antigen, immunoblotting and ELISA were carried out using polyclonal and monoclonal antibodies directed against EmAP and the Em2 antigen, respectively. In addition, the localization of EmAP and the Em2 antigen was compared by immunofluorescence and immunogold electron microscopy in *in vitro*-generated *E. multilocularis* metacestodes. The results show that common epitopes between EmAP and Em2 exist, which are predominantly of a peptidic nature. Both antigens are localized in an acellular parasite structure, the laminated layer, with additional locations for the EmAP on the glycocalyx and in the central region of invaginated protoscoleces. These results suggest a putative functional relationship between the two antigens and that Em2 could originate from EmAP.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Echinococcus multilocularis;* cestodes; alkaline phosphatase; Em2 antigen; AE, alveolar echinococcosis; AP, alkaline phosphatase; EmAP, *E. multilocularis* alkaline phosphatase; pNPP, *p*-nitrophenylphosphate.

Echinococcus multilocularis is a cestode parasite, whose larval stage causes alveolar echinococcosis (AE) and thus represents an ecologic and public health problem in many countries of the northern hemisphere. *E. multilocularis* metacestodes develop mainly in the liver of infected hosts and are characterized by tumor-like growth. They are composed of an acellular laminated layer followed by an inner cellular germinal layer and a fluid-filled cavity. Traditionally, investigations on *E. multilocularis* metacestodes have primarily involved animal experimentation. However, procedures for the long-term *in vitro* cultivation of these parasite larvae have been recently described (Hemphill and Gottstein 1995; Jura *et al.* 1996), with the advantage of eliminating the complex host influence. *E. multilocularis* metacestodes possess a high alkaline phosphatase (AP; EC 3.1.3.1) activity which has been previously purified and biochemically characterized (Sarciron *et al.* 1991; Lawton *et al.* 1995). The parasite enzyme was found to exhibit original properties compared to the corresponding enzyme from jird and sheep liver tissue: a several-fold increased activity, resistance toward heat denaturation, differences in response to various AP inhibitors, and slight differences in apparent molecular weight and in isoelectric point. In studying the last two criteria, we found that they matched exactly those previously published for the reference diagnostic antigen Em2 (Gottstein 1985). This antigen is currently used, in combination with another antigen, II/3-10 (Felleisen and Gottstein 1994), in a commercially available, reliable immunodiagnostic test for the specific diagnosis of AE. The Em2 antigen can be specifically detected using the MAb G11 (Deplazes and Gottstein 1991) and is not recognized by most of the sera from patients suffering from cystic echinococcosis, caused by the closely related *E. granulosus* (Gottstein *et al.* 1993). Since EmAP and Em2 apparently

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share the same isoelectric points and apparent molecular weight, we decided to further investigate the relationships between these two molecules using polyclonal and monoclonal antibodies directed against both antigens.

The Savoie strain of *E. multilocularis* was maintained in jirds (*Meriones unguiculatus*) by intraperitoneal passage every 3 months. The metacestodes were collected, cleared from host tissue, and frozen in liquid nitrogen until further use.

For the *in vitro* cultivation of *E. multilocularis* metacestodes, the cloned isolate KF5 was used (Gottstein *et al.* 1992). Cultures were maintained for up to 3 months as described previously (Hemphill and Gottstein 1995).

Alkaline phosphatase was purified from the metacestodes as previously described (Sarciron *et al.* 1991). Briefly, it was extracted from the minced metacestodes with *n*-butanol, followed by concanavalin A–Sepharose affinity chromatography. Elution was performed at 37°C according to Lawton *et al.* (1994, 1995) with 0.1 *M* α-methyl-mannoside/0.1% Triton X-100 and the concentrated prepurified fraction was further purified by gel filtration on a Sephacryl HR300 column (Pharmacia Biotech, Saclay, France).

The Em2 antigen was purified by affinity purification on solid-phase MAb G11 according to Deplazes and Gottstein (1991).

A polyclonal anti-EmAP antiserum was prepared by immunizing a rabbit according to Vaitukatis (1971) with purified EmAP. For some experiments including the immunolocalization experiments, the antibodies were purified on the relevant EmAP band according to Goldstein *et al.* (1986). The polyclonal rabbit anti-Em2 G11 antiserum was prepared by immunizing rabbits with Em2 G11 antigen affinity-purified on MAb G11.

The murine MAb G11 was prepared as previously described (Deplazes and Gottstein 1991).

The antigen samples were boiled in Laemmli buffer and run on 4–20% minigels at 125 V for 2 hr (Novex, San Diego, CA). The antigens were blotted onto nitrocellulose with a semidry apparatus (Pharmacia Biotech, Saclay, France) for 4 hr at 4°C. The strips were cut and blocked with 5% BSA in PBS before overnight exposure at 4°C to the antibodies diluted 1/200 to 1/1000 in the same buffer. Several controls were performed and included an irrelevant MAb, included preimmune rabbit serum, and omitted the first antibody. After washing in PBS–0.5% Tween, the bands were incubated with 1/500 peroxidase-conjugated anti-mouse γ chain (Jackson, Interchim, Montluçon, France) or anti-rabbit IgG (Sigma, L'Isle d'Abeau, France) for 2 hr at room temperature and visualized with 4-chloro-1-naphtol/H_2O_2. For deglycosylation experiments, the strips were processed for 1 hr in the dark at room temperature with 40 m*M* sodium periodate in 50 m*M* acetate buffer, pH 4.5, prior to the blocking. Control strips were incubated in the buffer alone.

For direct ELISA experiments, microplates (Immulon 3, Dynatech, Chantilly, VA) were coated with 50 μl of the purified EmAP in 0.1 *M* sodium bicarbonate buffer, pH 9.6, at a final concentration of 2.7 μg/ml. One hundred microliters of MAb G11 (1/100; 10 μg/ml) and affinity-purified polyclonal anti-EmAP (1/200) was added in PBS/1% BSA/0.05% Tween 20 and incubated for 1.5 hr at 37°C. Controls included an irrelevant MAb and preimmune rabbit serum and omitted the primary antibody. After washings in the same buffer, 200 μl of peroxidase-conjugated goat anti-rabbit IgG (Sigma) or goat anti-mouse γ chain antibodies (Jackson) diluted 1/4500 were added and incubated for 1 hr at 37°C. The reaction was revealed with OPD (Sigma) and visualized at 405 nm after 45 min at 37°C. For deglycosylation experiments, the plates were incubated at room temperature with 40 m*M* sodium periodate in 50 m*M* acetate buffer, pH 4.5, for 1 hr in the dark, prior to the addition of the primary antibodies. Controls were incubated in the buffer alone.

For sandwich ELISA, the microplates wells were coated with 50 μl of MAb G11 (1 ng/ml) diluted in the coating buffer. One hundred microliters of purified EmAP at 0.45 μg/ml was added. Binding of the enzyme was assessed with the relevant polyclonal anti-Em2 G11 antibody at a 1/1000 dilution, followed by the peroxidase conjugate as above. Controls included the use of *E. granulosus* crude butanol extract and omitted the purified EmAP. To evidence AP activity, the MAb G11-coated plates were processed as above, except that 200 μl of 50 m*M* pNPP in 1 *M* diethanolamine buffer, pH 9.0, was incubated at 37°C for 16 hr. The reaction was read at 405 nm.

LR-White embedding and resin polymerization of *E. multilocularis* metacestodes were carried out as described

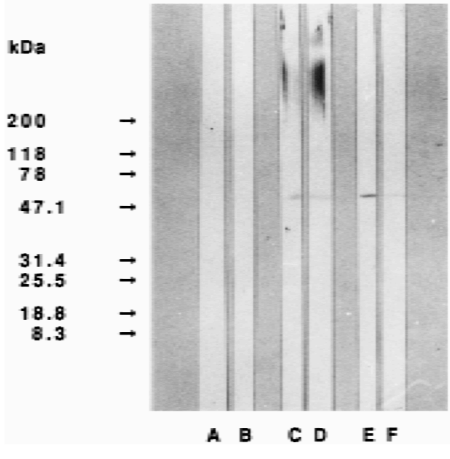


FIG. 1. Immunoblotting of the purified *Echinococcus multilocularis* AP 53-kDa monomer probed with the MAb G11 and the affinity-purified polyclonal anti-EmAP. (Lanes A, C, E) Control reduced EmAP. (Lanes B, D, F) Sodium periodate-treated EmAP. (Lanes A, B) Preimmune rabbit serum. (Lanes C, D) MAb G11. (Lanes E, F) Affinity-purified rabbit anti-EmAP.

previously (Hemphill and Gottstein 1995). Sections of 1–2 μm thickness were allowed to settle onto poly-L-lysine-coated coverslips, and nonspecific binding sites were blocked in PBS containing 1% (w/v) BSA and 50 m*M* glycine overnight at 4°C. All subsequent incubations were done at room temperature. Sections were incubated with the MAb G11 diluted 1/200 in blocking buffer for 1 hr, followed by an anti-mouse IgG conjugated to FITC (Sigma, St. Louis, MO) for 1 hr. Then, the affinity-purified rabbit anti-EmAP antibody was applied, followed by an anti-rabbit TRITC (Sigma, St. Louis, MO). After extensive washing in PBS, the sections were stained with the DNA-specific Hoechst 33258 (25 μg/ml) for 2 min. Finally, the preparations were briefly washed in distilled water and were embedded in a mixture of glycerol/gelvatol containing 1,4-diazobicyclo(2,2,2)octane (Merck, S.p.A., Milan, Italy) as an antifading reagent (Hemphill *et al.* 1992). Results were obtained with a Leitz Laborlux fluorescence microscope. Controls included incubation of sections (1) with preimmune serum and (2) with an affinity-purified rabbit anti-β-galactosidase antibody.

Ultrathin sections of LR-White embedded *E. multilocularis* metacestodes were loaded onto formvar/carbon-coated 200-mesh nickel grids and were incubated in EM blocking buffer (PBS containing 0.3% (w/v) BSA and 50 m*M* glycine) overnight at 4°C. The grids were then incubated with the affinity-purified anti-EmAP antibody for 1 hr, followed by buffer rinses (5× 5 min). The second layer (goat anti-rabbit conjugated to 10-nm gold particles; Amersham, Zürich, Switzerland) was incubated at a 1:5 dilution in PBS/0.1% BSA for 1 hr. Grids were washed in PBS and air-dried. They were then stained with lead citrate and uranyl

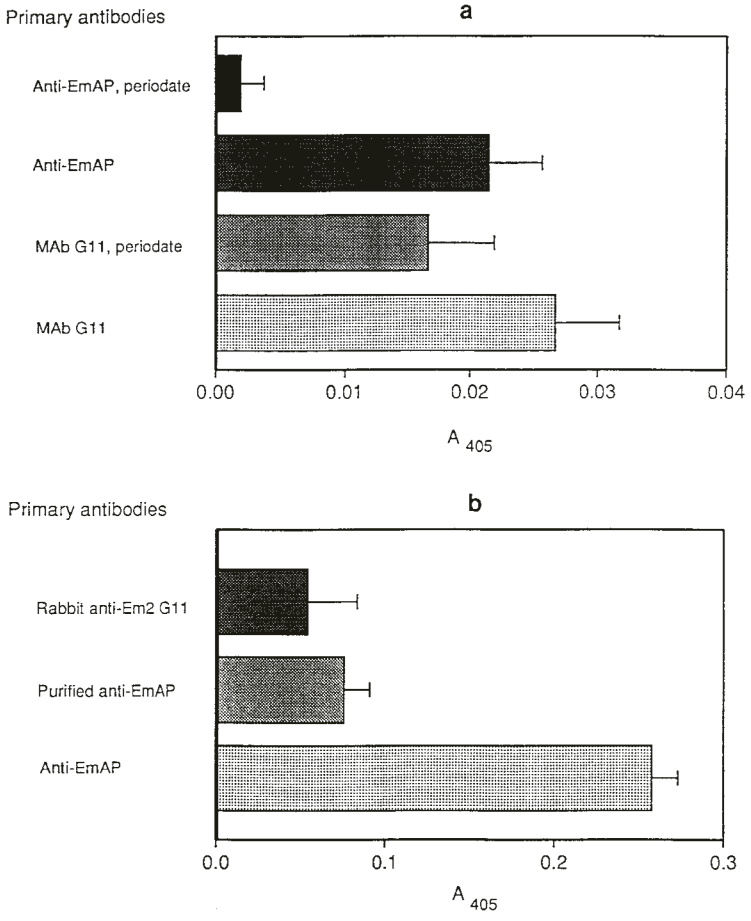


FIG. 2. (a) Direct ELISA with purified *Echinococcus multilocularis* AP-coated microplates. The control values for both the native and the deglycosylated enzymes were substracted. Values (mean ± 2 SD) account for three separate experiments. Affinity-purified anti-EmAP was used. (b) Sandwich ELISA with MAb G11-coated microplates. The data (mean ± 2 SD) refer to the testing of three different purified EmAP preparations. The control values were subtracted.

acetate (Hemphill and Croft 1997). The controls were the same as above. The preparations were inspected on a Philips Model 600 electron microscope operating at 60 kV.

Preliminary immunoblotting experiments gave evidence that MAb G11—as well as the polyclonal anti-Em2 antibodies (data not shown)—reacted with the purified EmAP. In order to define the nature of the epitopes recognized by these antibodies, SDS–PAGE-separated and nitrocellulose-bound purified EmAP was treated with sodium periodate. Chemical deglycosylation induced only a limited decrease in the banding intensity with the MAb G11. Conversely, the epitopes recognized by the rabbit polyclonal anti-EmAP showed a marked decrease in banding intensity; thus recognized epitopes were mainly carbohydrates (Fig. 1).

ELISA tests largely confirmed the above findings. Microplates were coated with purified EmAP. MAb G11 strongly reacted with the purified enzyme, with a slight difference after deglycosylation (Fig. 2a). A sandwich ELISA was performed using the MAb G11 as a capture probe. This experiment evidenced that purified EmAP from different preparations bound to the antibody (Fig. 2b). In addition, crude butanol extracts and purified EmAP bound to MAb G11-coated microplates (Fig. 3a) and were also revealed by alkaline phosphatase activity after incubation with the substrate (Fig. 3b).

To compare the localization of the two antigens, *in vitro*-generated *E. multilocularis* metacestodes were embedded in LR-White resin and the overall distribution of EmAP and Em2 was assessed by immunofluorescence using on-section double labeling with affinity-purified polyclonal anti-EmAP

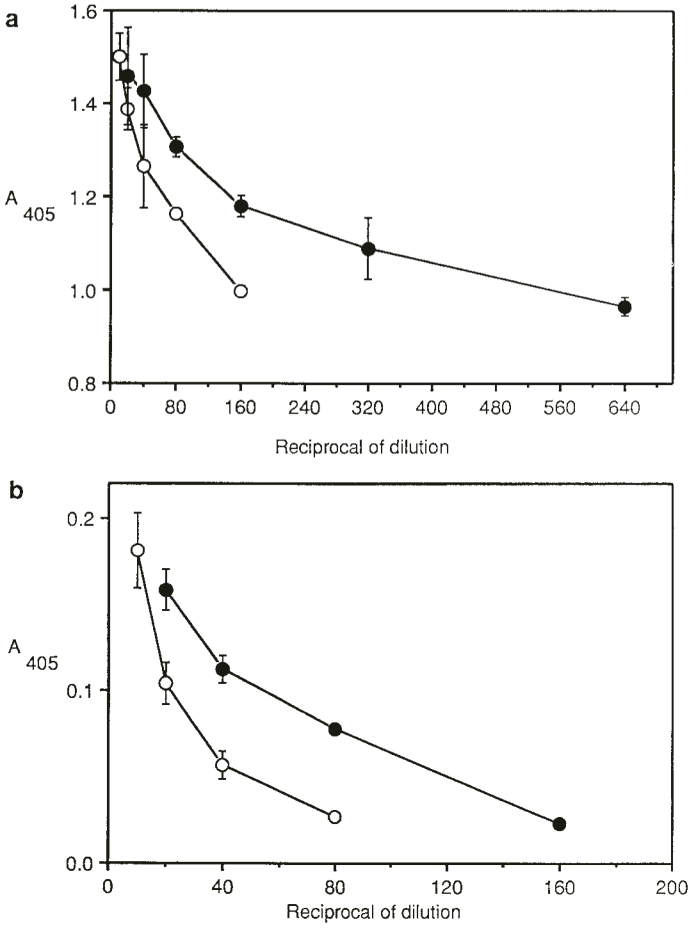


FIG. 3. (a) Sandwich ELISA. Serial dilutions of crude *E. multilocularis* butanol extract (●) and purified EmAP (○) were added to MAb G11-coated microplates. Results from three separate experiments. (b) Alkaline phosphatase activity of the EmAP captured by MAb-G11 coated onto the microplate. Serial dilutions of crude butanol extract (●) and purified EmAP (○) were added and the enzymatic activity was assayed after a 16-hr incubation at 37°C with the specific substrate, pNPP.

and the MAb G11 (Fig. 4). The localization of the Em2 antigen was strictly confined to the outer laminated layer of the metacestode. The EmAP was also present on the laminated layer, but was not confined to this structure. Labeling could also be observed on parts of the outer tissue as well as in the central region of the protoscoleces.

More defined information on the localization of EmAP was obtained by immunogold electron microscopy using the same LR-White-embedded parasites. As can be seen in Fig. 5, the affinity-purified anti-EmAP antibody bound strongly to the laminated layer of the metacestode wall, whereas labeling of the tegument, muscle cells, glycogen-storage cells, or undifferentiated cells was only marginal. Staining of the protoscoleces was restricted to the glycocalyx and the central region, whereas the integument was only marginally labeled.

The Em2 antigen was initially identified and characterized by Gottstein (1985). More recently, preliminary biochemical investigations have shown that the Em2 antigen, due to its resistance to proteinase K digestion and chloroform extraction, is likely to be largely composed of carbohydrates (Gottstein *et al*. 1994). The murine monoclonal antibody MAb G11 (Deplazes and Gottstein 1991) specifically reacts with an epitope present on the Em2 antigen of *E. multilocularis* metacestodes. Using *in vitro*-generated *E. multilocularis* metacestodes, it was shown by immunofluorescence and immunoelectron microscopical analysis that the Em2 antigen was a surface-bound molecule, primarily expressed in the laminated layer adjacent to the germinal layer of the metacestode (Hemphill and Gottstein 1995). Several investigations using both animal experimentations and *in vitro* cultivation of *E. multilocularis* metacestodes, protoscoleces, and oncospheres, as well as the analysis of AE patients' sera, suggested that the Em2 antigen plays an important protective role during infection and disease mediated by *E. multilocularis* (Gottstein *et al*. 1992, 1994; Gottstein and Felleisen 1995).

Purification of the *E. multilocularis* alkaline phosphatase and biochemical characterization provided evidence that this glycoprotein seems to be an important enzyme for the parasite, since its activity was reported to be 50 times greater than hepatic tissue AP of control and infected animals (Sarciron *et al*. 1991). The distinct differences from the corresponding host hepatic enzyme involved in the purification of EmAP, as well as its thermostability, have led to the conclusion that the parasite and the host hepatic enzymes were intrinsically different molecules (Sarciron *et al*. 1991; Lawton *et al*. 1995). Recently, it was shown that it may be used to reflect parasite viability and to monitor the course of infection and therapy of the disease (Sarciron *et al*. 1997). The biochemical characterization of the purified EmAP (Sarciron *et al*. 1991; Lawton *et al*. 1995) revealed that the apparent molecular weight of the enzyme monomer and its isoelectric point matched those originally reported for the Em2 antigen (Gottstein 1985). Thus this study was undertaken to characterize the relationships between the two antigens using polyclonal and monoclonal antibodies di-

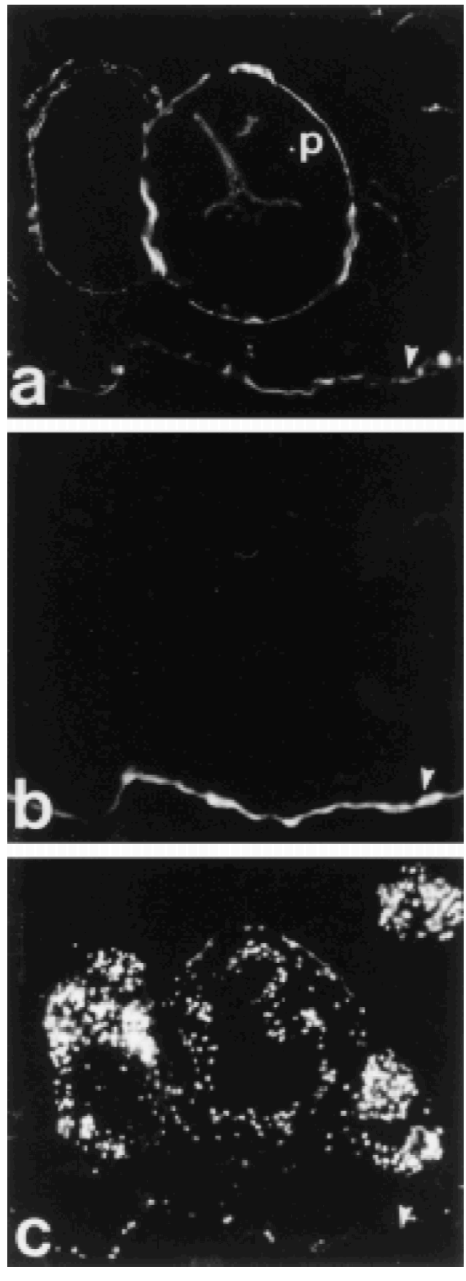


FIG. 4. Triple-immunofluorescence on-section staining of *in vitro*-generated *Echinococcus multilocularis* metacestodes embedded in LR-White resin. The same section was stained with (a) the affinity-purified rabbit anti-EmAP and a secondary TRITC-conjugated anti-rabbit IgG, (b) the MAb G11 specific for the Em2 antigen and a secondary FITC-conjugated anti-mouse IgG, and (c) the DNA-specific stain Hoechst 33258. Note the co-localization of the Em2 antigen and EmAP on the acellular laminated layer (arrowhead). p, brood capsule containing a protoscolex.

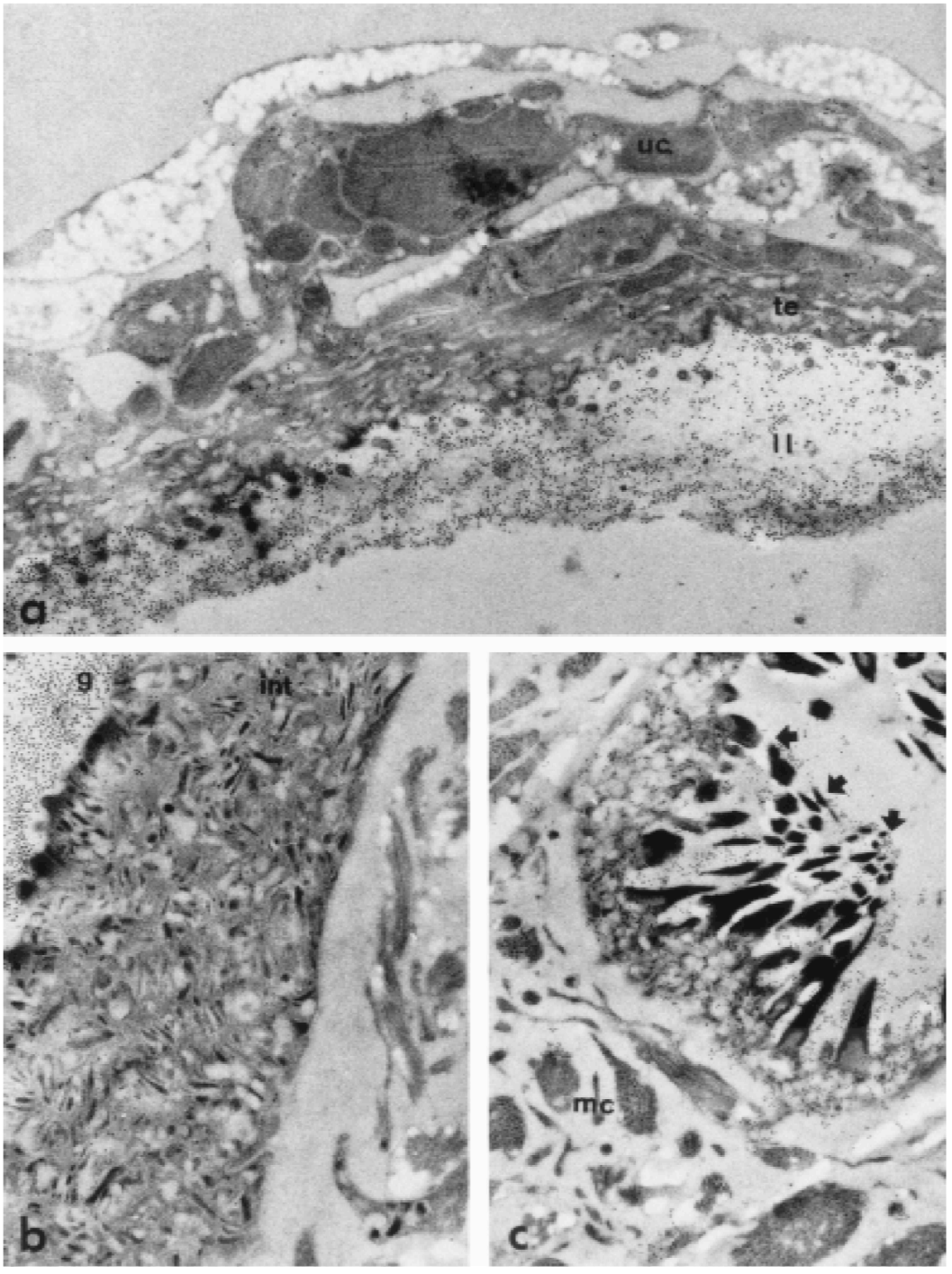


FIG. 5. Transmission electron micrographs of LR-White sections from the same parasite as shown in Fig. 4, immunogold labeled with the affinity-purified anti-EmAP antibody. (a) Micrograph of the metacestode wall: EmAP is abundant on the laminated layer of the metacestodes (ll), but is largely absent from the syncytial tegument (te) or the undifferentiated cells (uc) of the parasite tissue. (b) Micrograph of the peripheral region of the protoscolex: EmAP is abundant on the glycocalyx of the protoscoleces (g), but is largely absent from the integument (int) or any other part. (c) Central, still invaginated part of the protoscolex: the abundance of EmAP is demonstrated on the surface associated microtriches (arrows), whereas the neighboring cells are only marginally labeled. mc, muscles cells.

rected against EmAP and Em2. In order to rule out any potential contamination with host antigens, the exact localization of the two antigens was determined by on-section labeling of *in vitro*-generated LR-White-embedded *E. multilocularis* metacestodes.

Whereas EmAP has been extensively biochemically characterized (Sarciron *et al.* 1991; Lawton *et al.* 1995), the characterization of the Em2 antigen has largely focused upon its potential for immunodiagnosis, its localization, and its immunological role during host–parasite interplay (reviewed by Gottstein and Felleisen 1995; Gottstein and Hemphill 1996). This leaves the question of the biochemical nature of the Em2 antigen. Previous studies demonstrated that proteinase K digestion as well as chloroform extraction did not affect the reactivity of the purified Em2 antigen with the MAb G11. This indicated that this antigen could be largely of carbohydrate nature (Gottstein *et al.* 1994). The presence of carbohydrate residues on the Em2 antigen was confirmed by lectin-binding analysis, demonstrating that β-D-galactose, α-D-galNAc, β-D-gal(1-3)-D-galNAc, $(D\text{-}glcNAc)_2$, and NeuNAc are components of this molecule (Gottstein *et al.* 1994).

The immunoblotting experiments presented in this study showed that indeed, the purified EmAP was recognized by the MAb G11 as well as the rabbit polyclonal anti-EmAP antibody. In order to determine whether this reaction was based on the recognition of carbohydrate residues or involved protein epitopes, chemical deglycosylation was performed. This treatment of the carbohydrate part of the enzyme only slightly reduced the binding of the MAb G11, thus indicating that the involved epitope is mainly of protein origin.

ELISA assays were performed to rule out the possibility that MAb G11 binding did occur only because the enzyme was denatured, thus exposing epitopes which would not be accessible in the native form. Both MAb G11 and the rabbit anti-Em2 G11 antibody recognized the native form of the purified enzyme coated on the plates, confirming the specific binding of anti-Em2 antibodies to EmAP. Again, oxidative deglycosylation was quite ineffective in reducing the signal obtained with both the polyclonal and the monoclonal (MAb G11) anti-Em2 antibodies, indicating that these antibodies do react with a proteinaceous epitope(s). On the contrary, the reaction of the polyclonal anti-EmAP antibody was markedly inhibited after this treatment, showing that most of the recognized epitopes were of carbohydrate origin. In addition, MAb G11-coated microplates were incubated with butanol extracts and the purified EmAP. The presence of the captured enzyme was evidenced by measuring its activity, which showed that MAb G11 did indeed react with the purified EmAP.

The MAb G11 does indeed bind to epitopes present on EmAP that are mainly of proteinaceous origin. Since the Em2 antigen is insensitive to proteinase K and chloroform extraction, and thus apparently composed of carbohydrates (Gottstein *et al.* 1994), one must conclude that at least small peptides are also attached to the molecule and are common to EmAP. In addition, these results are consistent with the fact that the Em2 antigen does not bind to Con A (Gottstein *et al.* 1994), if one considers that the cross-reacting epitopes are not the carbohydrates. Since purification of EmAP involves Con A affinity chromatography, the sugar moieties involved, if any, do not originate from the Con A-specific part of the EmAP molecule.

The next experiments were performed to study and compare the localization of EmAP and the Em2 antigen. They were carried out on LR-White sections of *in vitro*-generated *E. multilocularis* metacestodes in order to rule out the possibility of nonspecific cross-reactions with interfering host components (Hemphill and Gottstein 1995). When metacestodes are grown in infected animals, host molecules are likely to be incorporated into the laminated layer through the surrounding connective tissue (Mehlhorn *et al.* 1983). Immunofluorescence double labeling demonstrated that the localization of the Em2 antigen was restricted to the outer portion of the metacestode. This confirmed earlier findings which demonstrated by immunogold electron microscopy that this antigen was an integral component of the acellular, carbohydrate-rich laminated layer (Hemphill and Gottstein 1995; Gottstein and Hemphill 1996). Staining with the affinity-purified anti-EmAP antibody provided evidence that EmAP co-localized with the Em2 antigen on the laminated layer. However, the anti-EmAP antibody also labeled other parts of the parasite, namely the peripheral and the central parts of the invaginated protoscoleces. The immunogold study clearly demonstrated that the enzyme was heavily enriched on the surface-associated structures of the parasite: on the laminated layer, on the glycocalyx of the protoscoleces, and on the centrally located, surface-exposed microtriches of the still invaginated protoscoleces. A logical hypothesis would be that EmAP and Em2 are closely related *in situ,* as well as metabolically, and that Em2 antigen could derive from EmAP. This could account for the more numerous structures labeled by the affinity-purified anti-EmAP antibody.

The fact that both Em2 and EmAP are predominantly localized on surface-associated structures, and thus on the regions of the parasite which interacts with the host, clearly indicates that these two antigens may play important roles in the host–parasite relationship. It is conceivable that the phosphatase activity expressed on the parasite surface may represent an important factor with respect to the host immune defense, especially with regard to host immune cell recognition. A functional relationship between these two antigens can be postulated and future studies will focus on elucidating the functional significance of both EmAP and the Em2 antigen in terms of host–parasite interactions.

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