Identification of a laminated layer-associated protein in *Echinococcus multilocularis* metacestodes

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SUMMARY

Echinococcus multilocularis is a cestode parasite that predominantly infects red and arctic foxes as definitive hosts. Ingestion of *E. multilocularis* eggs and subsequent post-oncospheral infection with the larval stage (metacestode) of the parasite results in alveolar echinococcosis (AE), a life-threatening hepatic disease concerning humans and other intermediate hosts such as small rodents. The primary fluid-filled vesicles of the asexually proliferating metacestode are comprised of an inner germinal layer, a syncytial tegument, and an outer, acellular, so-called laminated layer. This laminated layer may play an important role in protecting the developing *E. multilocularis* metacestode from host immune reactions, and laminated layer-associated components represent potential targets for intervention during the course of AE. We have used an *in vitro* cultivation technique for the long-term maintenance and proliferation of *E. multilocularis* metacestodes in order to generate premature (protoscolex-free) parasite vesicles. A polyclonal antiserum was raised against this host-free parasite tissue. Subsequent immunoblot analysis of parasite fractions obtained by Triton X-114 extraction lead to the identification of a 116 kDa protein (named EmP2) within the Triton-insoluble fraction. The characterization of EmP2 by SDS–PAGE, Western blotting, and by immunofluorescence revealed that EmP2 is a laminated layer-associated protein.

Key words: *Echinococcus multilocularis*, laminated layer, host–parasite interaction.

INTRODUCTION

Echinococcus multilocularis is a small tapeworm whose natural life-cycle involves predominantly red and arctic foxes as definitive hosts (Gottstein & Reichen, 1996). The hermaphroditic adult-stage tapeworm lives in the small intestine of its definitive host. Gravid proglottid uteri contain round to ovoid eggs (30–36 μm in diameter) each containing a single, fully differentiated oncosphere (Lethbridge, 1980). Such proglottids, and the free eggs released upon their rupture, are shed into the environment with the faeces. When ingested by a suitable intermediate host or accidentally by humans, digestive processes and other factors in the host gut result in hatching and release of the oncosphere. The oncosphere actively penetrates the epithelial border of the intestinal villi within 30–120 min (Lethbridge, 1980) and enters venous and lymphatic vessels to finally reach the liver where maturation to the asexually proliferating metacestode takes place (Sakamoto & Sugimura, 1970).

The continuous size increase and tumour-like growth of the metacestode causes finally the disease called alveolar echinococcosis (AE). AE is one of the most lethal helminthic infections in humans (reviewed by Gottstein & Hemphill, 1997). The ultrastructure of the metacestode tissue has been extensively investigated in several species of the genus *Echinococcus* (Mehlhorn, Eckert & Thompson, 1983; Delabre *et al*. 1987; Casado & Rodriguez-Caabeiro, 1989; Nahhas *et al*. 1991; Smith & Richards, 1991). It consists of an inner, germinal layer, followed by the tegument, a syncytial layer which surrounds the entire metacestode. The tegument plays an important role with respect to uptake of nutrients and excretion of waste material. Originating from the tegument, microtriches protrude outwards well into the outer, acellular, laminated layer whose components are synthesized within the tegument and secreted via these structures (Hemphill & Gottstein, 1995). Proliferation of the metacestode takes place by exogenous budding and results in progressive invasion of the surrounding tissue by the parasite (Eckert, Thompson & Mehlhorn, 1983).

Several lines of evidence obtained *in vivo* and *in vitro* indicate that the laminated layer may play an important role in protecting the developing *E. multilocularis* metacestodes from host immune reactions. (i) In field studies upon screening of human patients, it became evident that the laminated layer can remain within the infected host tissue for a long time, even after spontaneous dying-out of the larval parasite (Rausch *et al*. 1987; Lanier *et al*. 1987; Condon *et al*. 1988). (ii) Using immunofluorescence staining with the monoclonal antibody mAbG11 it was shown that the laminated layer-associated

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parasite carbohydrate antigen Em2 (Deplazes & Gottstein, 1991; Hemphill & Gottstein, 1995) was primarily expressed in oncospheres that started to synthesize the laminated layer within 2 weeks after egg-hatching *in vitro*. This is a period corresponding approximately to that required for a host to generate a specific systemic immune response (Gottstein, Deplazes & Aubert, 1992). (iii) No protection is achieved against established metacestodes already carrying the laminated layer (Gottstein *et al*. 1992). (iv) Protoscoleces lacking the laminated layer and the Em2 antigen cannot induce secondary AE in rodents (Gottstein *et al*. 1992). (v) Immunogold electron microscopical analysis of the host–parasite interface in infected mice demonstrated the direct physical interaction of host lymphoid cells with the laminated layer and the Em2 antigen (Gottstein & Hemphill, 1997).

More information is required in order to elucidate the mechanisms which enable *E. multilocularis* metacestodes to establish themselves within the host. Since the laminated layer represents the most outer surface of the parasite, the information needed could be acquired by molecular analysis of the laminated layer and its associated molecules, followed by studies on the specific interactions between these components with defined host immune and non-immune cells. Traditionally, respective investigations have involved predominantly animal experimentation (Kilejian & Schwabe, 1971; Persat *et al*. 1990). However, these studies were hampered by the fact that, within an infected animal, host material is likely to be incorporated, putatively in a form modified by the parasite, into the laminated layer as well (Mehlhorn *et al*. 1983). More recently, the methodology of *in vitro* cultivation of *E. multilocularis* metacestodes for the long-term maintenance and proliferation of parasites devoid of host cell components has been described (Hemphill & Gottstein, 1995, 1996*a*; Jura *et al*. 1996). We now report on the characterization of a laminated layer-associated protein which has been identified in *in vitro*-generated *E. multilocularis* metacestodes.

MATERIALS AND METHODS

Experimental design

In order to identify and characterize proteins which would be associated with the laminated layer of *E. multilocularis* metacestodes, the following experimental approach was used. *E. multilocularis* metacestodes were cultivated *in vitro*, and a selection of a specifically premature stage without protoscolex formation was made. Vesicle fluid was removed from the metacestode tissue, and a polyclonal rabbit anti-*E. multilocularis* antiserum was generated. Metacestode tissue was extracted employing the non-ionic detergent Triton X-114 (Sigma). The detergent-insoluble parasite fraction was characterized using the rabbit anti-*E. multilocularis* metacestode antiserum by SDS–PAGE and Western blotting. Monospecific antibodies were affinity purified out of the polyspecific rabbit antiserum on individual immunoreactive bands of potential relevance. Affinity-purified antibodies and the respective immunoreactive protein were characterized using biochemical and immunocytochemical techniques.

Biochemicals

If not otherwise stated, all reagents and tissue culture media were purchased from Gibco-BRL (Zürich, Switzerland).

In vitro *cultivation of* E. multilocularis *metacestodes*

In vitro cultivation of *E. multilocularis* metacestodes was carried out as described by Hemphill & Gottstein (1995). Briefly, C57BL/10 mice were infected intraperitoneally with a cloned isolate of *E. multilocularis* KF5 (Gottstein *et al*. 1992). After 2 months, the mice were euthanized, and the parasite tissue was recovered from the peritoneal cavity under aseptic conditions. This freshly recovered metacestode tissue was cut into small tissue blocks (0·5 cm^3) and was washed twice in Hanks balanced salt solution (HBSS). Two pieces of tissue were placed into 40 ml of culture medium (RPMI 1640 containing 12 mM HEPES, 10% FCS, 2 mM glutamine, 200 U of penicillin/ml, 200 μg of streptomycin/ml, 0·50 μg fungizone/ml). Alternatively, vesicle suspensions were prepared by mincing the tissue with a scalpel blade and pressing the residues through a sterile stainless-steel sieve (0·5 mm diameter mesh size). The small parasite vesicles which had passed the sieve were washed twice with HBSS and were resuspended in 40 ml of culture medium. Both vesicle suspension and tissue blocks were kept in tightly closed culture flasks (75 cm^2) placed in an upright position in an incubator at 37 °C, 5% CO_2, with medium changes every 2–4 days.

Isolation of in vitro*-generated metacestode vesicle walls and vesicle fluid*

Intact vesiculated metacestodes of 1–5 mm diameter were harvested from *in vitro* cultures after 2–5 weeks of cultivation. The time of vesicle collection was selected such as to exclude protoscolex formation which usually only occurs after several weeks of cultivation (Hemphill & Gottstein, 1995). Therefore, vesicles without protoscoleces were taken after 2–3 weeks of cultivation, while protoscoleces harbouring vesicles were harvested after 5 weeks. After 3 washes in distilled water, all the water was removed and the tube containing the vesicles was placed on ice. The

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metacestodes were then gently broken up using a pipette, and the preparation was centrifuged at 3000 ***g*** for 30 min at 4 °C. The supernatant fraction (containing vesicle fluid) and the pellet (representing the metacestode tissue) were carefully collected and stored at −80 °C before further use.

*Preparation of a rabbit anti-*E. multilocularis *antiserum*

A polyclonal antiserum directed against vesicle walls (anti-Em-vesicle) was generated in a rabbit. Prior to immunization, the pre-immune serum was tested by immunoblotting and immunofluorescence (see below) in order to ensure the absence of antibodies against *E. multilocularis* metacestodes. The animal was immunized by 3 successive subcutaneous injections of metacestode tissue from approximately 50 *in vitro*-cultivated vesicles each: once with metacestode tissue emulsified in Freund's complete adjuvant, and twice with vesicle tissue resuspended in Freund's incomplete adjuvant at days 10 and 20, respectively. The serum was collected at day 28, aliquoted and stored at −80 °C.

Extraction and fractionation of E. multilocularis *vesicle tissue employing the non-ionic detergent Triton X-114*

Metacestode tissue from approximately 100 *in vitro*-generated, protoscolex-free vesicles previously stored at −80 °C were resuspended in 6 ml of cold PBS (phosphate-buffered saline, pH 7·4). Phenylmethylsulfonyl fluoride (100 mM in ethanol, Sigma, Mo., USA) was added to make up 0·5 mM, and the suspension was incubated for 5 min on ice. Triton X-114 (1 %) was added and incubated on ice for 10 min with occasional vortexing. The preparation was centrifuged (200 ***g***, 5 min, 4 °C), and the supernatant and pellet were collected separately for further processing. The supernatant was centrifuged again (200 ***g***, 30 min, 4 °C), and the second supernatant (containing Triton X-114-soluble proteins) was incubated at 37 °C for 1 min. The hydrophilic and the hydrophobic phases were separated by a further centrifugation step (1000 ***g***, 2 min, room temperature), and samples for SDS–PAGE were processed using methanol/chloroform extraction (Wessel & Flügge, 1984). The pellet of the first centrifugation (containing detergent-resistant proteins) was washed twice with 3 ml of PBS, and was finally also processed for SDS–PAGE.

Immunoblotting, affinity purification of antibodies, and detection of carbohydrate residues

Samples of total *E. multilocularis* metacestode extracts or corresponding amounts of the different fractions were separated by SDS–PAGE. Gels were either stained by Coomassie blue or silver staining, or were electrophoretically transferred to nitrocellulose filters. In some experiments, the filters were treated with 50 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 5·5, for 30 min at room temperature in the dark. Blocking of unspecific binding sites was carried out for 4 h at 24 °C in PBS containing 3 % BSA (bovine serum albumin) and 0·3 % Tween-20. The antiserum directed against *E. multilocularis* metacestode tissue was applied at a dilution of 1:1000 in PBS/0·3 % BSA/0·3 % Tween-20 overnight at 4 °C. The filters were then washed 3 times in PBS/0·3 % Tween, and the bound antibodies were visualized using horse-radish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin antibodies (Promega, Madison, Wis., USA) according to the instructions provided by the manufacturers. As a control, nitrocellulose filters containing the same samples were incubated with pre-immune serum from the same rabbit.

Affinity-purified antibodies directed against specific immunoreactive bands of the P-fraction were prepared as described earlier (Hemphill & Gottstein, 1996*b*). Briefly, the bands corresponding to the immunoreactive proteins were cut out from nitrocellulose filters following preparative SDS–PAGE and Western blotting. After blocking of unspecific binding sites in PBS/3 % BSA/0·3 % Tween-20, the anti-*E. multilocularis* antiserum was applied at a dilution of 1:10 in PBS/0·3 % BSA/0·3 % Tween-20 overnight at 4 °C. After washing the strip in PBS/0·3 % Tween-20 three times for 10 min each, the bound antibodies were eluted in 750 μl of low pH buffer (50 mM Tris, 50 mM glycine, pH 2·6) for 5 min on ice with occasional vortexing. Then the strip was removed, and 75 μl of 1 M Tris base was immediately added. The eluted antibody fraction was centrifuged (10000 ***g***, 20 min), in order to remove small nitrocellulose particles with bound antigen, and BSA was added to the supernatant fraction to a final concentration of 0·1 %. Affinity-purified antibodies were aliquoted and stored at −20 °C. They were used for immunoblotting at a dilution of 1:20.

In order to identify potential carbohydrate moieties, the GlycoTrack™ Carbohydrate Detection Kit (Oxford GlycoSystems, Oxford, UK) was employed, following the procedures as recommended by the manufacturer. Glycoproteins were detected by incubation with a streptavidin–alkaline phosphatase conjugate followed by a colour reaction (Hemphill *et al*. 1997).

Immunocytochemistry

Immunofluorescent surface labelling of in vitro*-cultivated* E. multilocularis *metacestodes*. All procedures were carried out at room temperature. *In vitro*-

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generated mature metacestode vesicles (0·5–1 mm diameter), collected at a stage where protoscolex formation had occurred, were washed 3 times in PBS and were placed into wells of an ELISA plate. They were incubated in IF-blocking buffer (PBS containing 0·5 % BSA and 50 mM glycine) for 2 h. After 3 additional washes in PBS, vesicles were labelled for 1 h with either anti-*E. multilocularis* antiserum (anti-EM-vesicle) (1:250 in IF-blocking buffer), the corresponding pre-immune serum (1:250), affinity-purified anti-EmP2 antibody (undiluted), or an irrelevant affinity-purified rabbit antibody (anti-Nc-p43, undiluted, hemphill (1996)). The antibodies were removed and the vesicles gently washed 3 times for 5 min in PBS. The secondary antibody was a goat anti-rabbit-IgG conjugated to FITC (Sigma) diluted 1:100 in IF-blocking buffer. After 1 h, the secondary antibody conjugate was removed, the vesicles were gently washed in PBS (3 times, 5 min), and the specimens were inspected using a Leitz DM IL inverted fluorescence microscope.

Fixation and embedding for post-embedding immunocytochemistry. For immunocytochemical localization of EmP2 and the Em2-antigen on sections of *E. multilocularis* metacestodes, either *in vitro*-generated vesicles or small blocks (1 mm^3) of infected tissue freshly removed from the peritoneal cavity of a C57BL/10 mouse, were embedded in LR-White resin (Sigma). Fixation took place in 100 mM phosphate buffer containing 3 % paraformaldehyde/0·1 % glutaraldehyde for 40 min at 4 °C. Metacestode tissue was then rinsed twice in PBS, and free aldehyde groups were saturated with 50 mM glycine in PBS for 1 h at room temperature. After 3 washes in PBS, specimens were dehydrated through 50, 70 and 90 % ethanol for 10 min each, and 3 washes in 100 % ethanol for 15 min each. All dehydration steps were carried out at −20 °C. Metacestodes were infiltrated with 4 changes of LR-White during 48 h at −15 °C. The resin was then polymerized at 60 °C for 8–12 h.

Indirect immunofluorescence of LR-White sections. Sections 1 μm thick of LR-White embedded metacestode tissue were cut on a Reichert and Jung ultramicrotome, loaded onto poly-L-lysine coated cover-slips and air dried. Sections were incubated overnight in PBS supplemented with IF-blocking buffer at 4 °C. They were then rinsed in PBS, and incubated for 1 h with the affinity-purified anti-EmP2 antibody or with an irrelevant affinity-purified anti-Nc-p43 antibody (both undiluted). After rinsing the specimen in PBS, the cover-slips were incubated for 45 min in FITC-conjugated goat anti-rabbit antibody (1:100 in IF-blocking buffer). Samples were washed in PBS and were subsequently incubated with mAbG11 (Deplazes & Gottstein, 1991) diluted 1:100 in IF-blocking buffer. The fourth antibody layer was a goat anti-rabbit-IgG conjugated to TRITC (Sigma). Subsequently, the cover-slips were washed in PBS (6 times, 5 min) before embedding in a mixture of gelvatol/glycerol containing 1,4-diazobicyclo (2.2.2) octan (Merck AG, Zürich). They were viewed on a Leitz Laborlux S fluorescence microscope.

RESULTS

Characterization of crude antigen fractions and hyperimmune serum

A polyclonal antiserum directed against *in vitro*-cultivated *E. multilocularis* metacestode tissue was generated in a rabbit. The reactivity of the anti-*E. multilocularis* antiserum was primarily assessed by immunofluorescent staining of the surface of intact, *in vitro*-cultivated vesicles. This yielded bright labelling all over the parasite surface (Fig. 1 A, B).

Metacestodes were biochemically fractionated using an extraction procedure employing the non-ionic detergent Triton X-114. This resulted in 3 biochemically defined fractions: (i) detergent-insoluble components, (ii) soluble hydrophilic proteins, and (iii) detergent-soluble hydrophobic molecules (Fig. 2, lanes 1–3). The different fractions were tested on immunoblots stained with the anti-*E. multilocularis* antiserum (Fig. 2, lanes 4–6). As other batches of *in vitro*-cultivated parasites were thus processed, the pattern of immunoblot staining obtained in these 3 biochemically defined fractions was always very similar (data not shown). Incubations with the corresponding pre-immune serum did not result in any staining, neither by immunoblotting nor by immunofluorescence assays (data not shown).

Affinity-purification of antibodies

In order to obtain specific antibodies for the characterization of potential laminated layer-associated proteins, we performed affinity purifications on the most prominent reactive bands of the fraction which contained proteins resistant towards Triton X-114 extraction. These proteins had apparent molecular weights of 44, 100, 116 and 200 kDa (see Fig. 2). The affinity-purified antibodies were subsequently tested by SDS–PAGE and immunoblotting for their specificity towards the bands against which they had been selected. Only 1 of these antibodies, that affinity-purified on the 116 kDa band designated EmP2, was shown to be specifically directed against its respective protein (Fig. 2, lane 7). In addition, anti-EmP2 antibody also recognized a protein band of identical molecular weight within the fraction containing hydrophilic detergent-

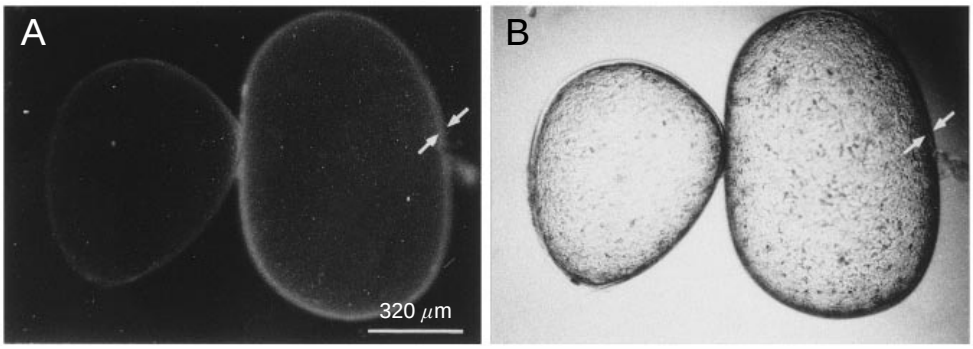

Fig. 1. Immunofluorescence whole-mount labelling using anti-*Echinococcus multilocularis* antiserum. (A) Surface labelling of *in vitro*-generated metacestodes employing the anti-*E. multilocularis* antiserum and FITC-conjugated secondary antibodies. Note the distinct labelling of the laminated layer (arrows). (B) Corresponding phase-contrast image is shown.

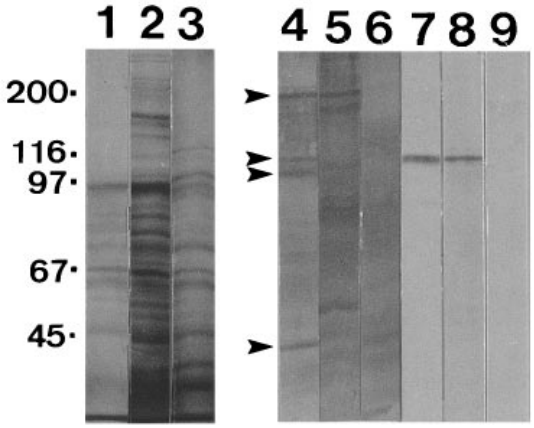

Fig. 2. Affinity purification and characterization of anti-EmP2 antibodies. Lanes 1–3 demonstrate the different *Echinococcus multilocularis* fractions obtained after extraction from Triton X-114, separation by SDS–PAGE and silver staining. Lane 1, pellet after Triton X-114 extraction; lane 2, hydrophilic Triton X-114-soluble proteins; lane 3, hydrophobic proteins. Lanes 4–6 are the corresponding Western blots stained with the anti-*E. multilocularis* antiserum. Lanes 7–9 are corresponding Western blots stained with the affinity-purified anti-EmP2 antibody. Arrowheads mark those bands which were used for affinity purification. Molecular weight markers (kDa) are indicated.

soluble proteins (lane 8), but immunoreactivity was absent from the hydrophobic detergent-soluble protein fraction (Fig. 2, lane 9).

Characterization of EmP2

In order to determine whether anti-EmP2 antibodies react with proteinaceous or carbohydrate epitopes, chemical deglycosylation of SDS–PAGE separated and nitrocellulose-bound proteins of detergent-insoluble fractions was performed using sodium periodate treatment prior to incubation with the respective antibodies. The reactivity of affinity-purified anti-EmP2 antibodies with the 116 kDa protein remained unaffected (not shown). In addition, the presence of glycoproteins within the 3 *E. multilocularis* fractions was assessed using the GlycoTrack™ carbohydrate detection kit. While carbohydrates were present mainly within the detergent-insoluble fraction, no carbohydrate residues could be detected on a band of 116 kDa corresponding to EmP2 (data not shown).

The affinity-purified anti-EmP2 antibodies were tested for their reactivity with the laminated layer of the parasite. By immunofluorescent whole-mount surface labelling of intact *in vitro*-generated vesicles, incubations with anti-EmP2 antibody resulted in rather intense labelling of the surface of these vesicles (Fig. 3A, B). On-section staining of LR-White-embedded *in vitro*-cultivated metacestodes was also performed. Triple fluorescence labelling of metacestodes collected after 3 weeks (no protoscolex formation, see Fig. 3C–E) and 8 weeks (containing brood capsules, see Fig. 3F–H) using (i) affinity-purified anti-EmP2 antibodies, (ii) the monoclonal antibody (mAb) G11 which is directed against the laminated layer antigen Em2 (Deplazes & Gottstein, 1991), and (iii) the fluorescent dye Hoechst 23558 which indicates the presence of nuclei, showed that EmP2 displayed a very defined, localized distribution, near the interface between the acellular laminated layer and the actual parasite tissue, the tegument (Fig. 3C, F). This was in contrast to the Em2 antigen (Fig. 3D, G), where staining was evenly distributed all over the laminated layer. In metacestodes which were collected at later time-points, anti-EmP2 staining could also be observed on the wall of the brood capsules and to some extent on the surface of the protoscoleces.

Sections of LR-White-embedded tissue blocks originating from infected C57BL/10 mice (exhibiting no protoscolex formation; Gottstein, Wunderlin & Tanner, 1994) were also investigated in terms of the localization of EmP2 by immunofluorescence. In contrast to what was observed for *in vitro*-generated metacestodes, anti-EmP2 staining within these *in vivo*-generated metacestodes was not predominantly

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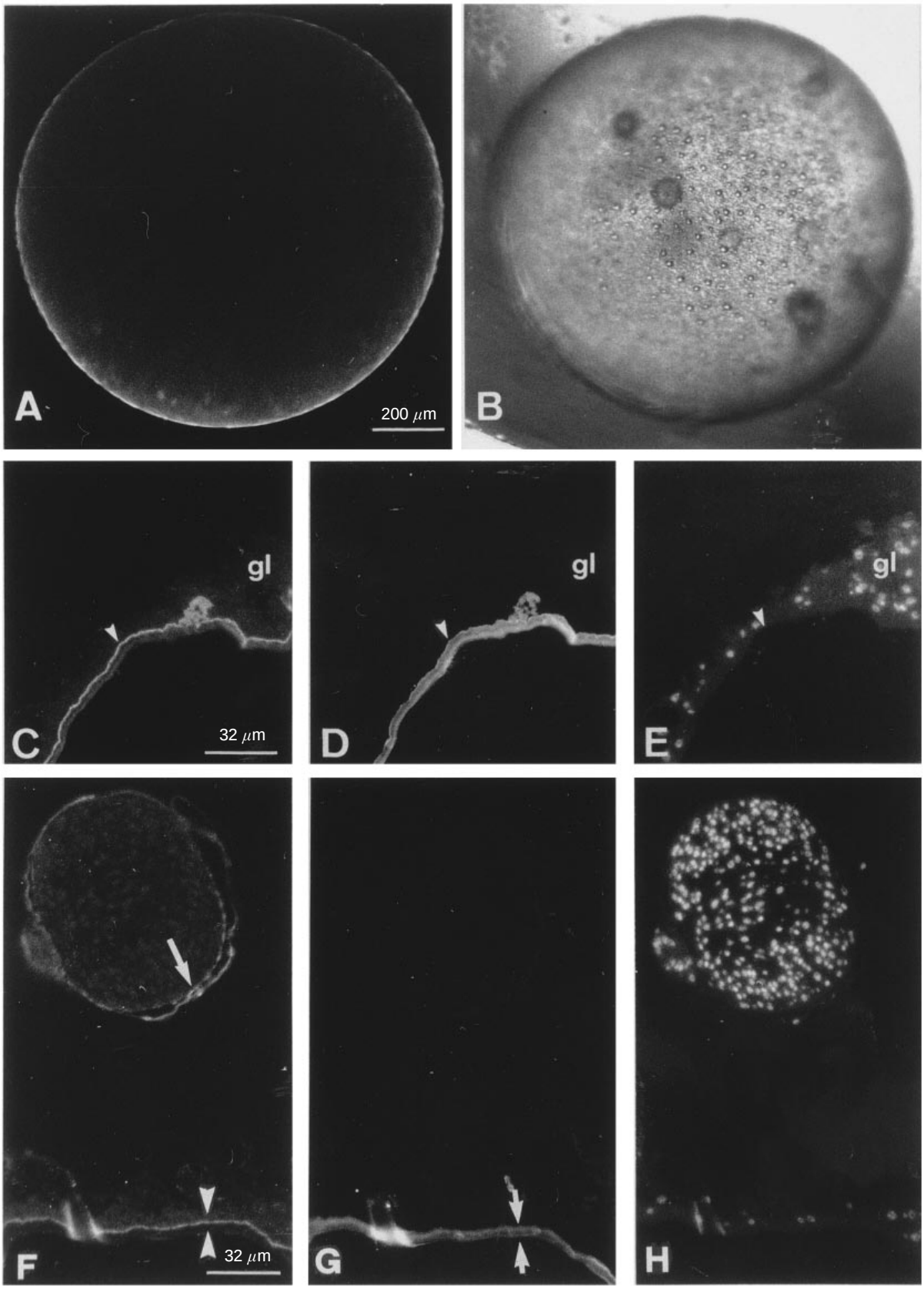

Fig. 3. Characterization of anti-EmP2 reactivity by immunofluorescence microscopy. (A and B) Whole-mount surface labelling of *in vitro*-generated *Echinococcus multilocularis* metacestodes using affinity-purified anti-EmP2 antibodies. (C–D) Triple fluorescence staining (C = anti-EmP2, D = mAbG11, E = Hoechst 23558) of a section through an LR-White embedded, *in vitro*-cultivated metacestode devoid of brood capsules (3 weeks of cultivation). Note the absence of EmP2 from the germinal layer of the parasite (gl) and its abundance at the tegument–laminated layer boundary (arrowheads). (F–H) Triple fluorescence staining as in (C)–(E) of a section through a metacestode harbouring a brood

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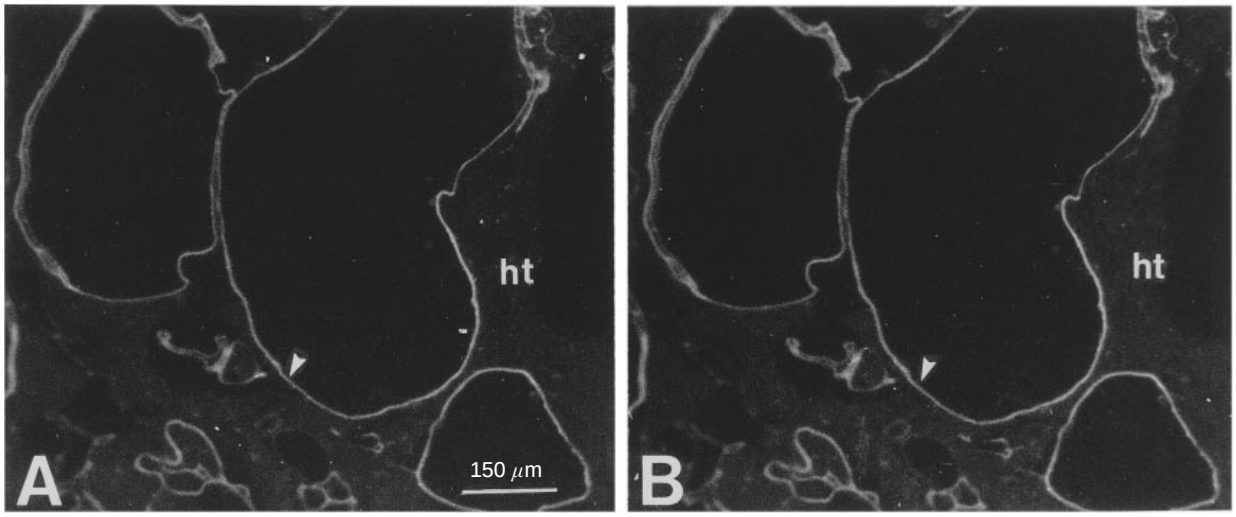

Fig. 4. EmP2 in mouse tissue infected with *Echinococcus multilocularis* metacestodes. Double immunofluorescence of EmP2 (A) and the Em2 antigen (B) in tissue of an infected C57BL/10 mouse. Note the identical staining pattern on the laminated layer of the parasite obtained with both antibodies. ht = Host tissue. The arrowheads mark the laminated layer.

enriched at the laminated layer–tegument boundary, but appeared to be evenly distributed along the entire laminated layer identically as for the Em2 antigen (Fig. 4).

DISCUSSION

The aim of the studies presented in this paper was to identify intrinsic components of the laminated layer of *E. multilocularis* metacestodes. The search for such laminated layer-associated proteins is necessary because the laminated layer may play an important role in protecting the developing *E. multilocularis* metacestode from host immune reactions (Rausch *et al*. 1987; Condon *et al*. 1988; Gottstein, 1992; Gottstein *et al*. 1992, 1994; Gottstein & Felleisen, 1995). Elucidation of the composition of this acellular, parasite-derived structure could lead to a better understanding of the events occurring at the host–parasite interface, involving both host immune and non-immune components.

A critical first step in our studies was the isolation of suitable parasite tissue. Generally, the isolation of metacestodes from infected laboratory animals is not directly straightforward, since the parasite is always found to be intermingled with host connective tissue and infiltrating host immune and non-immune cells. It is therefore very likely that, in infected animals, host components are incorporated into the acellular laminated layer at the host–parasite interface (Mehlhorn *et al*. 1983; Delabre *et al*. 1987; Casado & Rodriguez-Caabeiro, 1989; Nahhas *et al*. 1991). Furthermore, parasite expressed antigens may become modified divergently by different intermediate hosts and may, in this way, render the search for original antigens difficult. For these reasons, the experiments were carried out on parasite tissue obtained from *in vitro*-cultivated *E. multilocularis* metacestodes (Hemphill & Gottstein, 1995, 1996*a*). The parasites obtained from our *in vitro* cultures were completely devoid of host components which could cause unspecific results or variation within different tissue batches, and we were able to monitor the developmental stages such as to obtain either premature, protoscolex-free vesicles, or mature, differentiated metacestodes carrying brood capsules and protoscoleces.

A polyclonal antiserum against *E. multilocularis* metacestode tissue was raised in a rabbit. Immunofluorescence whole-mount surface labelling of metacestodes demonstrated that the polyclonal antiserum did indeed contain immunoglobulins directed against the laminated layer. Thus, we expected that the anti-*E. multilocularis* antiserum would be a useful tool for the identification of laminated layer-associated proteins.

Since the acellular laminated layer of *E. multilocularis* metacestodes was previously demonstrated to be a structure which is immunologically and physiologically inert (Gottstein & Felleisen, 1995; Gottstein & Hemphill, 1997) it was assumed that this layer, or most of its components, were also

capsule with developing protoscoleces. Note the specific staining of affinity-purified anti-EmP2 antibodies on both the tegument–laminated layer boundary (arrowheads) and the surface of the brood capsule (large arrow), while the Em2 antigen is strictly confined to the laminated layer (small arrows).

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relatively resistant towards Triton X-114 detergent extraction. Using antibodies affinity-purified on immunoreactive bands of a Triton X-114-insoluble fraction we identified a 116 kDa protein named EmP2. Immunoblotting demonstrated that affinity-purified anti-EmP2 antibodies also recognized a protein band of identical apparent molecular weight within the detergent-soluble hydrophilic fraction, while no immunoreactivity could be detected within the fraction containing hydrophobic proteins. This could indicate that 2 populations of EmP2 could exist: (i) a population which would be associated to a particular, detergent-resistant structure such as the laminated layer and (ii) another detergent-soluble, hydrophilic population.

Our investigations on the distribution of EmP2 are in favour of this hypothesis. On one hand, immunofluorescence surface labelling of *in vitro*-generated metacestodes clearly showed that EmP2 was accessible from the outside, and thus most likely represents an intrinsic component of the laminated layer of *E. multilocularis* metacestodes. On the other hand, investigations on the intravesicular distribution of EmP2 showed that the distribution of this 116 kDa protein was clearly enriched at the interface between tegument and laminated layer. This indicates that EmP2 could originate from the parasite interior (i.e. the tegument) and would then be transported, via the microtriches, towards the peripheral areas of the parasite. Once this molecule has reached the laminated layer it could then be released as a hydrophilic, soluble protein. In addition, EmP2-specific staining was also observed on peripheral structures of the brood capsules which contain the developing protoscoleces. Similar findings were obtained for *E. multilocularis* alkaline phosphatase. However, this enzyme has recently been shown to be abundant on the entire laminated layer of the metacestode wall, on the glycocalyx of the protoscoleces, and also on the central, still invaginated microtriches of the protoscoleces within the brood capsules (Lawton *et al*. 1997). Preliminary investigations using immunoblotting have shown that EmP2 is also present within *E. multilocularis* vesicle fluid generated *in vitro* (Ingold *et al*. unpublished observations) and further work is in progress to show whether EmP2 is a component of the excreted/secreted fractions of *E. multilocularis* metacestodes.

In contrast to the immunostaining observed when anti-EmP2 antibodies were used, Em2-specific labelling was distributed on the entire laminated layer, confirming previous findings (Hemphill & Gottstein, 1995). Immunofluorescent labelling of LR-White-embedded metacestode tissue sections isolated from an infected C57BL/10 mouse using anti-EmP2 and anti-Em2 antibodies also showed that EmP2 was confined to the laminated layer of the parasite. In addition, this also indicated that EmP2 expression was not an artefact of *in vitro* cultivation, but that this protein was indeed also abundant in metacestodes generated in animals. In agreement to previous findings (Nakaya, Nakao & Ito, 1997), neither brood capsules nor protoscoleces could be found within these infected C57BL/10 mice. However, a distinct difference to the labelling pattern of *in vitro*-generated metacestodes was that EmP2 staining now appeared to be equally distributed all over the laminated layer, just as for the Em2 antigen. The reason for this is currently unknown. A possible explanation could be that the 2 types of metacestode tissues (*in vitro* and *in vivo*) investigated in this study are not directly comparable. They could represent different developmental stages and probably they also vary in their degree of differentiation.

Besides the Em2 antigen (Deplazes & Gottstein, 1991), not many other proteins have been identified to date to be localized within the laminated layer of *E. multilocularis* metacestodes. Frosch *et al*. (1993) reported the cloning of an echinococcal microtrichal antigen of 47 kDa molecular weight which was immunoreactive in *E. multilocularis* disease. The fact that a non-glycosylated protein such as EmP2 represents an intrinsic component of the laminated layer is somewhat surprising, since this structure has been shown to be largely constituted of carbohydrate residues (Kilejian & Schwabe, 1971; Gottstein, 1985; Gottstein *et al*. 1994). Employing lectin-binding studies, the Em2 antigen as a major component of this acellular layer was found to be composed of b-D-galactose, (a)-D-galNAc, b-D-gal(1-3)-D-galNAc, (D-glcNAc)2 and NeuNAc (Gottstein *et al*. 1994). In addition, *E. multilocularis* alkaline phosphatase is also a highly glycosylated protein (Lawton *et al*. 1997).

In conclusion, *in vitro* cultivation of *E. multilocularis* metacestodes has proven to be a very useful basis for our study in that we were able to identify and characterize the laminated layer-associated protein EmP2 using biochemical and immunological tools. Its localization at the host–parasite interface indicates that EmP2 could play a role during the interaction of the metacestode surface with adjacent host immune or non-immune cells. More detailed studies on the molecular level are under way in order to further characterize this protein and to elucidate its functional significance during host–parasite interactions in AE.

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