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CHARACTERIZATION OF THE LAMINATED LAYER OF IN VITRO CULTIVATED *ECHINOCOCCUS VOGELI* METACESTODES

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ABSTRACT: The metacestode (larval) stages of the cestode parasites *Echinococcus vogeli* and *E. multilocularis* were isolated from the peritoneal cavity of experimentally infected C57BL/6 mice and were cultured in vitro for a period of up to 4 mo under conditions normally applied for the in vitro cultivation of *E. multilocularis* metacestodes. In contrast to *E. multilocularis*, *E. vogeli* did not exhibit extensive exogenous budding and proliferation but increased in size with a final diameter of up to 10 mm. Most metacestodes contained protoscoleces, singly or in groups, either associated with brood capsules or growing directly out of the germinal layer. Each individual metacestode was covered by an acellular translucent laminated layer that was considerably thicker than the laminated layer of *E. multilocularis* metacestodes. The ultrastructural characteristics, protein content, and carbohydrate composition of the laminated layer of in vitro cultivated *E. vogeli* and *E. multilocularis* were assessed using transmission electron microscopy, lectin fluorescence labeling, and lectin blotting assays. The laminated layer of *E. vogeli* is, as previously described for *E. multilocularis* metacestodes, largely composed of N-acetyl- β -D-galactosaminyl residues and α - and β -D-galactosyl residues, as well as of the core structure of O-linked carbohydrate chains, N-acetylgalactosamine- β -1,3-galactose. However, in contrast to *E. multilocularis*, N-linked glycopeptides and α -D-mannosyl and/or glucosyl residues were also associated with the laminated layer of *E. vogeli*. The laminated layer from both species was isolated from in vitro cultivated metacestodes, and the purified fractions were comparatively analyzed. The protein:carbohydrate ratio (1:1) was similar in both parasites; however, the protein banding pattern obtained by silver staining following sodium dodecyl sulfate polyacrylamide gel electrophoresis suggested intrinsic differences in protein composition. A polyclonal antiserum raised against the *E. multilocularis* laminated layer and a monoclonal antibody, G11, directed against the major *E. multilocularis* laminated layer antigen Em2 did not cross-react with *E. vogeli*, indicating distinct compositional and antigenic differences between these 2 parasites.

Echinococcus species are common small tapeworms of domesticated and wild carnivores. The metacestode larvae may occur in humans, and many domestic and wild mammals, including rodents. The larvae may develop in various organs, causing dramatic damage depending on localization and lesion size. *Echinococcus* includes 4 recognized species: *E. multilocularis*, *E. granulosus*, *E. oligarthrus*, and *E. vogeli* (Thompson and Lymbery, 1995).

Echinococcus multilocularis is the causative agent of alveolar echinococcosis (AE), a disease prevalent in many areas of the Northern Hemisphere (Gottstein, 1992). The final host is the red fox (*Vulpes fulva*), although occasionally dogs and cats can also be involved. Eggs are shed into the environment with the feces. On ingestion, eggs pass through the stomach and then hatch, releasing oncospheres. Oncospheres penetrate the intestinal wall, reach blood and lymphatic vessels, and are mainly targeted to the liver. There, the oncosphere develops into the metacestode stage, which is characterized by fluid-filled vesicles of various sizes. *Echinococcus multilocularis* metacestodes proliferate by asexual exogenous budding, progressively invading and destroying the surrounding tissue (Mehlhorn et al., 1983). Occasional metastasis formation into other organs can occur.

Adult *E. vogeli* are found in bush dogs and in domesticated dogs; pacas (Rodentia: *Cuniculus paca*) serve as intermediate hosts (Rausch et al., 1981). Geographically, *E. vogeli* is restricted to the northern half of South America (Meneghelli et al., 1992). Occasionally, humans may be infected (D'Alessandro, 1997; Basset et al., 1998). The metacestodes of *E. vogeli* form primary polycystic hepatic abscesses that often extend deep into the parenchyma. Occasionally, metastasis formation into other

organs can occur. The cysts can be up to 8 cm in diameter, and growth and proliferation take place by 2 processes. Endogenous proliferation results in a large multicystic metacestode with several cavities. Each cavity is surrounded by a thin acellular laminated layer. Such *E. vogeli* cysts are normally surrounded by a fibrous capsule, similar to the adventitia of *E. granulosus*. Exogenous proliferation, which is probably more important in terms of pathogenicity, is associated with an invasive potential similar to that found for *E. multilocularis* (Rausch and D'Alessandro, 1999).

A common feature of all *Echinococcus* species is the laminated layer, which forms the outer surface of the parasite. This acellular structure is largely composed of carbohydrates and exhibits extreme physiological and immunological stability (Rausch et al., 1987; Lanier et al., 1988; Deplazes and Gottstein, 1991). This layer is the only metacestode structure that is in constant contact with the surrounding host tissue and most likely represents an important barrier against immunological and physiological reactions on part of the host (Gottstein and Hemphill, 1997). This layer also is 1 of the major sources presenting and delivering antigens to the host and thus must play a crucial role during infection. Nothing is known about the molecular characteristics of the laminated layer in *E. vogeli*. In the present study, we compared the ultrastructure and the molecular composition of this important structure in *E. vogeli* and *E. multilocularis* metacestodes cultured in vitro.

MATERIALS AND METHODS

Biochemicals

Unless otherwise stated, all reagents and tissue culture media were purchased from Gibco-BRL (Zürich, Switzerland).

In vitro culture of *E. multilocularis* and *E. vogeli* metacestodes

In vitro culture of *E. multilocularis* metacestodes was carried out as described by Hemphill and Gottstein (1995). Gerbils (*Meriones unguiculatus*) were infected intraperitoneally (i.p.) with a cloned isolate of *E. multilocularis* KF5 (Gottstein et al., 1992). After 1–2 mo, the gerbils

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were euthanized with CO₂, and the parasite tissue was recovered from the peritoneal cavity under aseptic conditions. The tissue pieces were cut into small tissue blocks (0.5 cm³) and washed in Hanks' balanced salt solution (HBSS). Six to 12 pieces of tissue were placed into 50 ml of culture medium (RPMI 1640 containing 12 mM HEPES, 10% fetal calf serum, 2 mM glutamine, 200 µg/ml penicillin, 200 µg/ml streptomycin, 0.50 µg/ml fungizone). Tissue blocks were kept in tightly closed culture flasks (75 cm²) placed in an upright position in an incubator at 37 C in 5% CO₂, with medium changes every 2–4 days. Metacystodes of *E. vogeli* (Rausch and D'Alessandro, 1999) were inoculated into the peritoneal cavity of C57BL/6 mice. After 4 mo, mice were euthanized, and metacystodes 1–3 mm in diameter were aseptically removed and washed with HBSS; 40–50 vesicles were placed into culture medium and cultured in vitro as for *E. multilocularis*.

Isolation of metacystode tissue

Intact vesiculated metacystodes were harvested from in vitro cultures after 2–3 mo of cultivation (Hemphill and Gottstein, 1995). Vesicle fluid was separated from the metacystode tissue as previously described (Ingold et al., 1998, 2000), and the tissue was either processed for ultrastructural investigations or lectin cytochemistry and immunohistochemistry or was stored at –80 C for subsequent biochemical analysis.

Scanning and transmission electron microscopy

Freshly harvested parasites were fixed in 2.5% glutaraldehyde/0.25% tannic acid (Sigma Chemical Co., St. Louis, Missouri) in 100 mM sodium phosphate buffer (pH 7.2) at 4 C for 3 hr and were postfixed in 2% osmium tetroxide (Sigma) for another 3 hr at 4 C. Samples were then extensively washed in distilled water and were incubated in 1% uranyl acetate for 2 hr at 4 C.

For scanning electron microscopy (SEM), metacystodes were dehydrated in acetone and were dried by sublimation in Peldri II (Plano GmbH, Marburg, Germany) as previously described (Hemphill and Gottstein, 1995). Specimens were placed onto glass coverslips, sputter coated with gold, and inspected on a Jeol 840 scanning electron microscope operating at 25 kV.

For conventional transmission electron microscopy (TEM), parasites were dehydrated in increasing concentrations of ethanol and infiltrated with Epon 812 resin (Plano), and the resin was polymerized overnight at 60 C (Hemphill and Croft, 1997). Sections 80–90 nm thick were cut on an ultramicrotome (Reichert and Jung, Vienna, Austria), placed onto 200-mesh copper grids, stained with uranyl acetate and lead citrate as previously described (Hemphill and Croft, 1997), and viewed on a Philips 300 transmission electron microscope operating at 60 kV.

Lectins

The following 8 biotinylated lectins (Sigma) were used. Concanavalin A (ConA) has a high affinity for *N*-linked carbohydrate chains and for alpha-D-mannosyl and alpha-D-glucosyl residues. Soybean agglutinin (SBA) and *Dolichos biflorus* agglutinin (DBA) bind to D-*N*-acetylglucosamine. *Tetragonolobus purpurea* agglutinin (TPA) and *Ulex europaeensis* agglutinin-I (UEA-I) exhibit a high specificity for alpha-D-fucosyl residues. Wheat germ agglutinin (WGA) binds to D-*N*-acetylglucosamine dimers and sialic acid residues, and *Artocarpus integrifolia* agglutinin (Jacalin) has a high affinity for the core structure of O-linked carbohydrate chains, *N*-acetylglucosamine-beta-1,3-galactose. Peanut agglutinin (PNA) binds specifically to D-galactosyl residues.

Antibodies

A polyclonal rabbit antiserum was directed against the purified laminated layer of in vitro generated *E. multilocularis* metacystodes (Ingold et al., 2000). Prior to immunization, the preimmune serum was tested by immunoblotting and immunofluorescence to ensure the absence of anti-*E. multilocularis* antibodies. The animal was immunized by 3 successive subcutaneous injections of purified laminated layer from approximately 50 vesicles cultured in vitro: once with laminated layer emulsified in Freund's complete adjuvant and twice with material emulsified in Freund's incomplete adjuvant at days 10 and 20, respectively. The serum was collected at day 28, aliquoted, and stored at –80 C. The monoclonal antibody (mAb) G11, directed against the major carbohydrate antigen of *E. multilocularis* metacystodes, has been previously

described (Deplazes and Gottstein, 1991; Hemphill and Gottstein, 1995).

Immunohistochemistry and lectin cytochemistry

Processing of metacystode tissue for embedment in LR-White resin (Sigma) for subsequent immunohistochemistry and lectin cytochemistry was performed as previously described (Ingold et al., 2000). For cytochemical localization of lectin binding sites on sections of *E. multilocularis* metacystodes, all steps were performed at room temperature. Sections were incubated for 2 hr with IF blocking buffer (phosphate-buffered saline [PBS] containing 1% bovine serum albumin [BSA] and 50 mM glycine). They were then rinsed in PBS and incubated for 1 hr with either 20 µg/ml of biotinylated lectins or 20 µg/ml of lectins plus 250 mM of the corresponding inhibitory sugars, all diluted in lectin binding buffer (20 mM Tris, 145 mM NaCl, 1 mM Mn²⁺, 1 mM Ca²⁺, 1 mM Mg²⁺, pH 7.4). Prior to use, the lectins and the inhibition control mixtures were centrifuged (10,000 g, 3 min) to remove possible aggregates and were incubated in a water bath for 1 hr at 37 C. After rinsing the specimens in PBS, the coverslips were incubated for 45 min in PBS containing 10 µg/ml streptavidin–fluorescein isothiocyanate (FITC; Sigma). Samples were then washed in PBS, incubated in the DNA-specific fluorescent dye Hoechst 32558 (1 µg/ml in PBS) for 2 min, and subsequently embedded in a mixture of glycerol/gelvatol containing 1.4-diazobicyclo[2,2,2]octan (Merck, Germany) as an antifading reagent. Samples were viewed on a Leitz Laborlux S fluorescence microscope.

For antibody labeling, sections of LR-White-embedded parasites were incubated in IF blocking buffer and then with rabbit antiserum directed against the purified laminated layer of *E. multilocularis* metacystodes at a dilution of 1:200 in PBS/0.5% BSA or with the mAb G11 directed against the major laminated layer-associated carbohydrate antigen of *E. multilocularis* metacystodes (Hemphill and Gottstein, 1995). After washing in PBS, the secondary antibody conjugates, either goat anti-rabbit FITC or goat anti-mouse FITC (Sigma), respectively, were applied at a dilution of 1:100 for 30 min. Following extensive washing in PBS, specimens were embedded as above.

Isolation of the laminated layer from in vitro cultivated metacystodes

The laminated layers of *E. multilocularis* and *E. vogeli* were isolated as described by Ingold et al. (2000). To each tissue pellet, corresponding to approximately 50 metacystodes 3–5 mm in diameter, 500 µl of 6 M urea in PBS was added. Samples were then incubated for 15 min at room temperature while being extensively vortexed. Subsequently, the preparations were centrifuged at 3,000 g for 20 min at 20 C. The supernatants, containing mostly cellular material and other extractable components, were removed. The insoluble material was washed twice with 1 ml PBS. The final pellets were solubilized in 1 ml PBS containing 6 M guanidium HCl and dialyzed into PBS overnight at 4 C. Protein concentrations were determined using the Biorad protein assay. The carbohydrate content of the samples was assessed by a modified orcinol-sulphuric assay (White and Kennedy, 1986). Two hundred microliters of sample and 800 µl of reagent (2 mg/ml resorcinol in concentrated sulphuric acid) were mixed, heated to 80 C for 15 min, and cooled to 20 C. Optical densities were read at 490 nm and referred to a standard curve prepared with 12.5–400 µg/ml Dextran T-2000 (Pharmacia, Uppsala, Sweden).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, lectin blotting, and immunoblotting

Characterization of purified laminated layer fractions was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and by subsequent western blot analysis. Equal amounts of *E. multilocularis* and *E. vogeli* laminated layer fractions were loaded, and the gels were either stained with silver or the periodic acid–Schiff (PAS) reaction (Thornton et al., 1996) or were electrophoretically transferred to nitrocellulose filters. Two western blotting techniques were used: a standard technique using a transfer buffer containing 5 mM Tris, 187 mM glycine, 20% methanol, pH 8.3, with the transfer filter arranged on the anodic side of the gel (Towbin et al., 1979) and an acidic blotting technique, employing a transfer buffer composed of 0.7% acetic acid in H₂O, conferring a positive charge to

the polypeptides and the nitrocellulose filter arranged on the cathodic side (Hames, 1990).

For the detection of carbohydrate moieties on proteins following separation by SDS-PAGE and transfer to nitrocellulose, the Glyco Track Carbohydrate Detection Kit (Oxford GlycoSystems, Oxford, U.K.) was employed, following the procedures recommended by the manufacturer (Ingold et al., 2000).

Lectin-binding sites on glycoproteins following SDS-PAGE and transfer to nitrocellulose were detected after application of both standard and acid blotting techniques. The same biotinylated lectins and corresponding inhibition controls as for lectin fluorescence studies were used, except that the lectins were diluted to 5 µg/ml in lectin binding buffer. Prior to incubation, western blots were blocked in TBST (Tris-buffered saline, pH 7.4, containing 0.3% Tween-20) for 2 hr. After an incubation of 1 hr at room temperature, the strips were washed 3 times for 10 min each in TBST before applying a streptavidin-alkaline phosphatase conjugate (PharMingen; 1:500 in PBS) for 45 min. The strips were washed 3 times for 5 min each in TBST and 3 times for 5 min each in lectin binding buffer before bound lectins were visualized by a color reaction.

For immunoblotting, nitrocellulose filters were blocked for 2 hr in TBST/3% BSA. Filters were then labeled with a polyclonal rabbit antiserum directed against the purified laminated layer of *E. multilocularis* metacystodes diluted 1:500 in TBST/0.3% BSA for 1 hr and were subsequently incubated in a goat anti-rabbit alkaline phosphatase conjugate (Promega; 1:500 in TBST) for 1 hr. Bound antibodies were visualized by a color reaction.

RESULTS

In vitro culture of *E. multilocularis* and *E. vogeli* metacystodes

The in vitro culture and respective morphology of *E. multilocularis* metacystodes have been previously described (Hemphill and Gottstein, 1995, 1996). In vitro culture is normally initiated by small pieces of parasite-infected tissue, and the cultures are characterized by extensive proliferation of parasite vesicles through exogenous budding from tissue blocks, followed by the formation of protoscoleces after several weeks of cultivation. In contrast, *E. vogeli* metacystodes obtained from infected C57BL/6 mice were primarily masses of vesicles of various diameters (0.5–4 mm) within the peritoneal cavity, often detached and proliferating individually. Approximately 50% of all vesicles had undergone differentiation and had already initiated protoscolex formation at the beginning of in vitro culture. During the in vitro culture period of 4 mo, *E. vogeli* metacystodes did not exhibit extensive proliferation, but they did increase in size (8–10 mm in diameter). In addition, over 90% of all metacystodes harbored protoscoleces after this cultivation period. Approximately 20 parasites were reintroduced into the peritoneal cavity of a C57BL/6 mouse. After 4 mo, these metacystodes had undergone extensive proliferation within the infected animal (data not shown), thus proving the maintenance of infectivity.

Inspection of metacystodes by SEM after in vitro culture for 3 mo revealed that *E. vogeli* metacystodes exhibited an overall morphology very similar to that of *E. multilocularis*. The 2 surfaces of the parasite, i.e., the outer acellular laminated layer and the inner germinal layer, could be easily distinguished (Figs. 1, 2). Larger metacystodes contained numerous brood capsules arising from the germinal layer (Fig. 3) with various numbers of protoscoleces, either invaginated or already evaginated (Figs. 4, 5). Frequently, protoscoleces were seen to protrude directly out of the germinal layer tissue (Fig. 6).

However, one striking morphological feature that discriminates *E. vogeli* metacystodes from those of *E. multilocularis* is

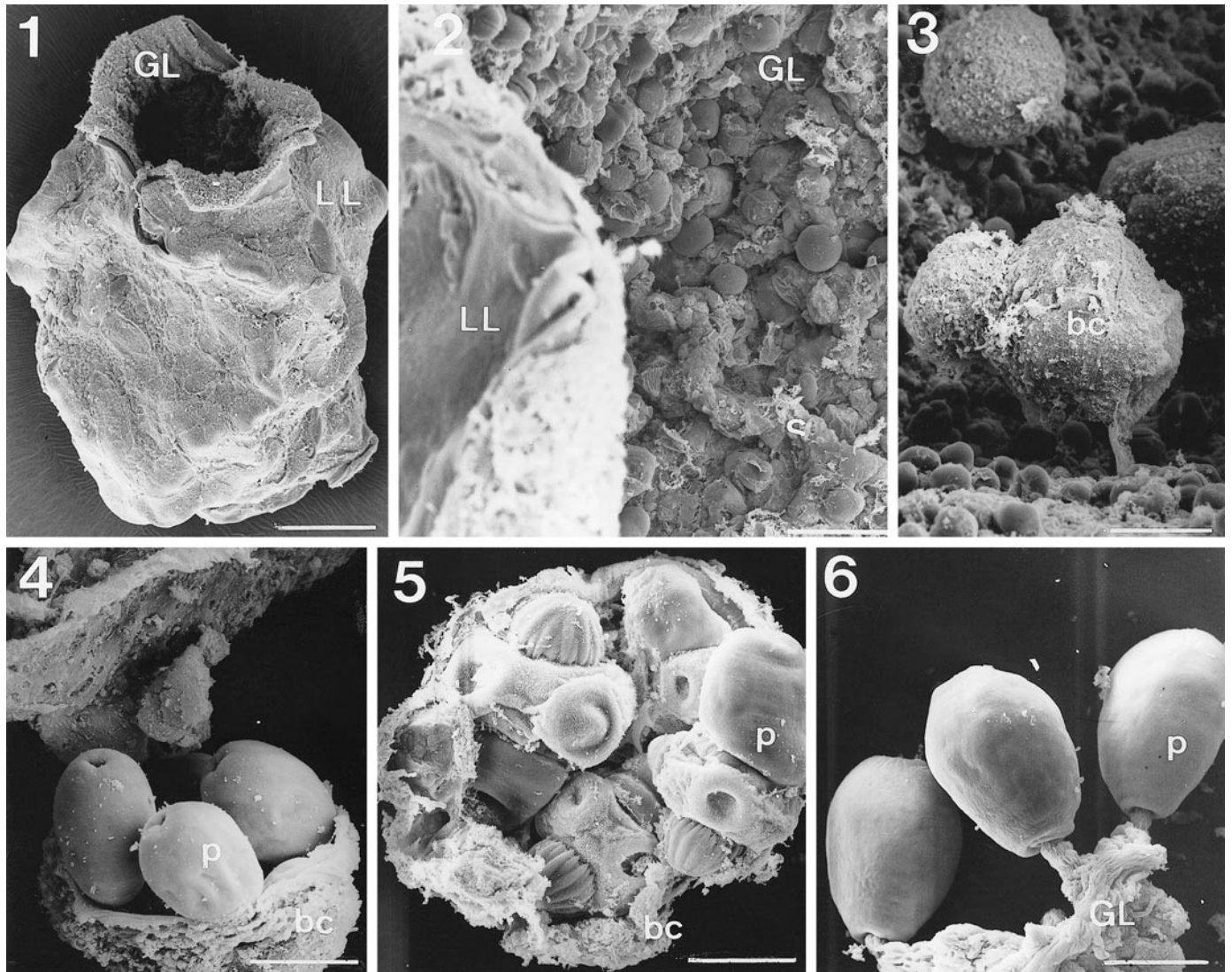
the thickness of the acellular laminated layer, i.e., 20–40 µm in *E. vogeli* and 5–10 µm in *E. multilocularis* metacystodes. Furthermore, the laminated layer of *E. vogeli* metacystodes appeared rather smooth and nonstructured as compared with the rougher, more textured surface of *E. multilocularis* metacystodes (Figs. 7, 8). Upon inspection by TEM, using a fixation protocol that incorporated tannic acid for improving the structural preservation of carbohydrate-based structures, the laminated layer of *E. vogeli* metacystodes exhibited an amorphous appearance, with small vesicles occasionally incorporated into its matrix (Figs. 9, 10). In contrast, TEM of *E. multilocularis* metacystodes revealed a distinct microfibrillar pattern (Figs. 11, 12). Other parasite features such as microtriches, tegument, and cells of the germinal layer appear structurally rather similar in both species.

Characterization of the laminated layer of *E. vogeli* metacystodes

Because the laminated layer of all *Echinococcus* species is most likely rich in carbohydrates, the lectin binding capacity of the laminated layer of *E. vogeli* metacystodes was assessed histochemically using lectin fluorescence labeling. Sections of in vitro cultured and LR-White-embedded parasites were incubated with a panel of 8 biotinylated lectins, followed by a streptavidin-FITC conjugate. ConA, SBA, WGA, PNA, and Jacalin bound strongly to the laminated layer of *E. vogeli* metacystodes (Figs. 13–22), and binding of these lectins was entirely inhibited in the presence of 250 mM of the corresponding inhibitory sugars. DBA, UEA-I, and TPA did not exhibit any staining (data not shown). Binding to the laminated layer was not uniform. ConA, SBA, and WGA bound most efficiently to the proximal part of the laminated layer more closely to the germinal layer, whereas WGA and Jacalin staining was more or less uniformly distributed over the entire structure.

To isolate this laminated layer from *E. vogeli* metacystodes for further biochemical analysis, we employed a procedure previously developed for the isolation of the same structure in *E. multilocularis* (Ingold et al., 2000). The laminated layer fraction was further analyzed with respect to protein and carbohydrate content. The protein:carbohydrate ratio was roughly 1:1 in the laminated layer fractions of both parasites (*E. multilocularis*: 140 µg/ml:130 µg/ml; *E. vogeli*: 1,420 µg/ml:1,470 µg/ml). However, separation of equal amounts of these fractions by SDS-PAGE and subsequent Coomassie and silver staining revealed clearly distinct protein banding patterns (Fig. 23). Carbohydrates on SDS-PAGE-separated fractions were detected using PAS staining. Although the purified laminated layer fraction of *E. multilocularis* metacystodes was enriched in bands migrating at a high molecular mass (>200 kDa), no high-molecular-mass glycans could be detected in the laminated layer fraction of *E. vogeli* metacystodes (Fig. 23A, B).

Transfer to nitrocellulose was carried out following SDS-PAGE employing conventional and acidic blotting techniques. Performing the blotting procedure in a solution containing 0.7% acetic acid in H₂O (Fig. 23C, D) resulted in a high-efficiency transfer of *E. multilocularis* high-molecular-mass molecules, whereas no PAS staining could be observed for *E. vogeli*. The *E. multilocularis* PAS-positive band also reacted with the antiserum directed against the laminated layer. If electrophoretic blotting was carried out in



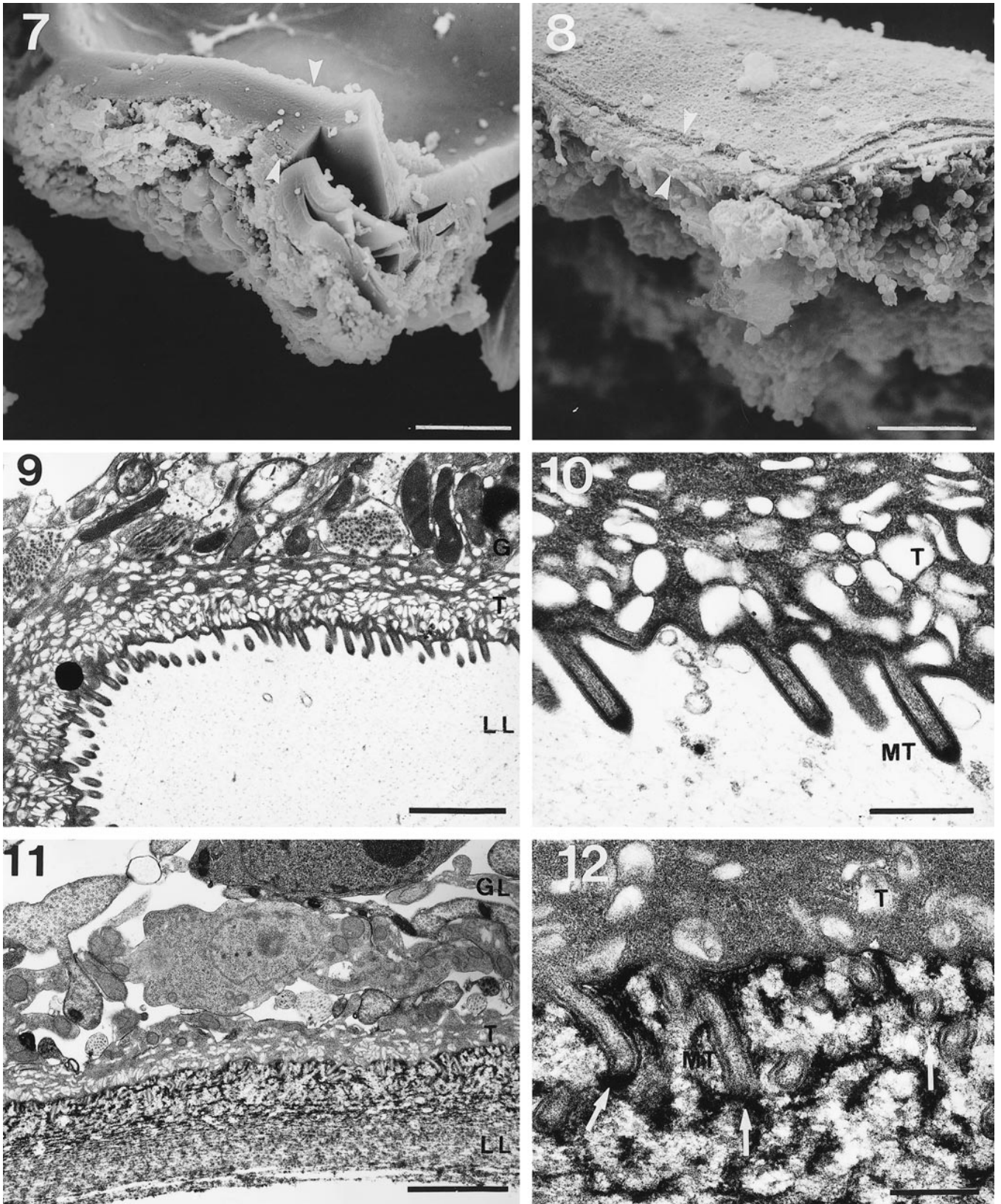
FIGURES 1–6. Scanning electron microscopy of *in vitro* cultivated *Echinococcus vogeli* metacestodes. **1.** Whole metacestode, with the outer laminated layer (LL) and the inner germinal layer (GL) clearly visible. Bar = 1.2 mm. **2.** Higher magnification view of the LL and the GL. Bar = 160 μm . **3–5.** Brood capsules (bc) arising from the germinal layer contain either (4) invaginated protoscoleces (p) or (5) already evaginated protoscoleces, with rostellum and suckers clearly visible. Bars = 180 μm (3), 130 μm (4, 5). **6.** Occasionally, protoscoleces can be seen arising directly from the germinal layer. Bar = 120 μm .

a conventional transfer buffer at pH 8.3 (Fig. 23E, F), the PAS-positive high-molecular-mass bands in *E. multilocularis* fractions were not efficiently transferred to the nitrocellulose filters and thus were not visible. However, the corresponding antiserum strongly labeled lower molecular mass proteins, but no cross-reactivity with *E. vogeli* was observed (Fig. 23E, F).

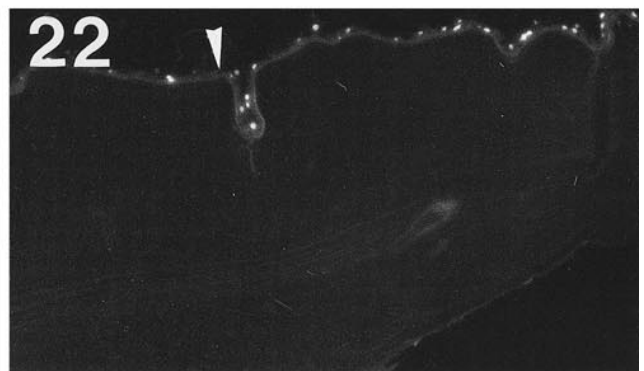
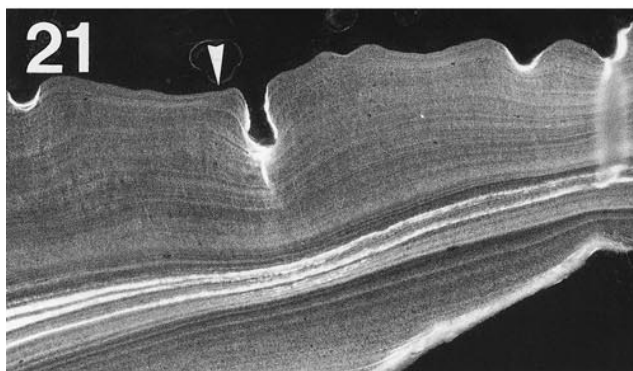
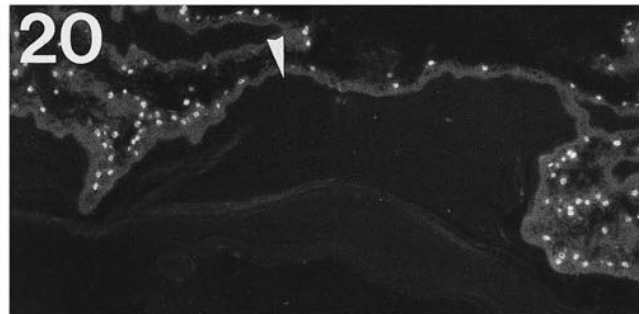
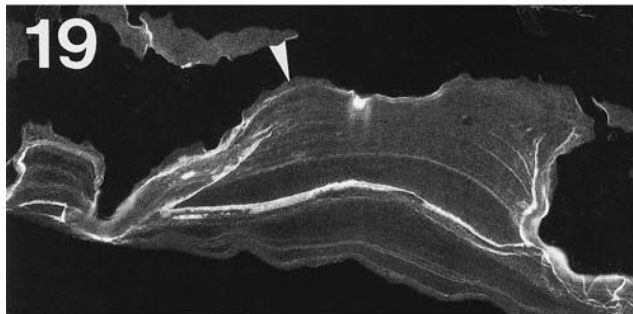
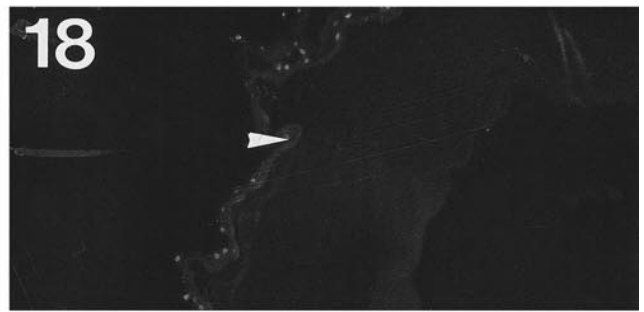
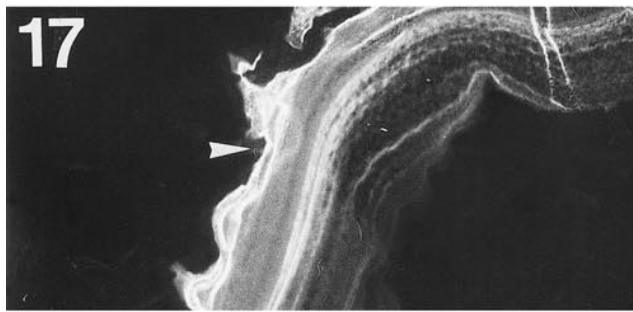
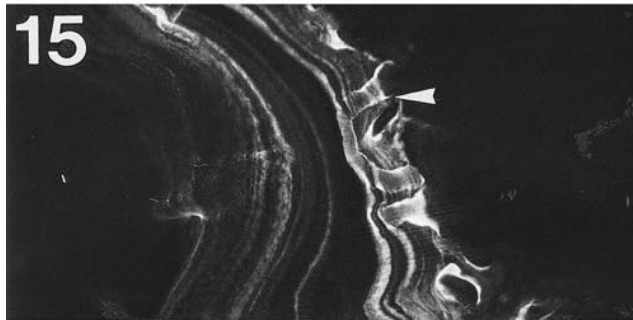
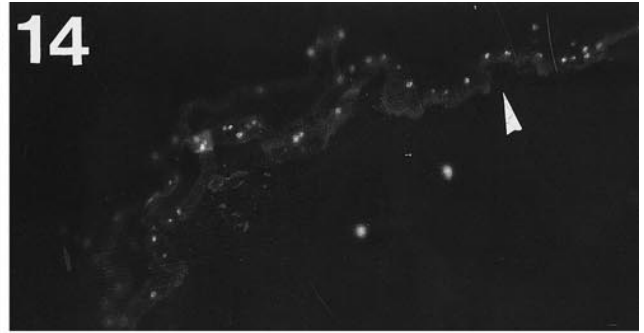
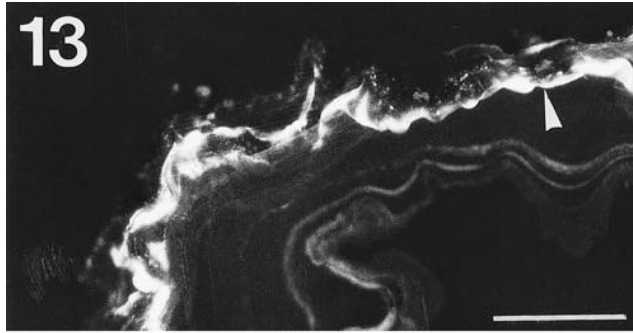
These differences were also observed with immunofluorescence staining of *E. multilocularis* and *E. vogeli* metacestodes using the anti-*E. multilocularis* laminated layer antiserum (Figs. 24–27). In addition, the mAb G11, directed against the major carbohydrate antigen of *E. multilocularis* metacestodes, did not exhibit any cross-reactivity with *E. vogeli* (Figs. 28–31).

Using both standard and acid transfer buffer, western blots of the purified laminated layer were prepared and assessed for lectin binding employing the same panel of 8 biotinylated lectins previously used for lectin fluorescence labeling (Fig. 32).

For *E. multilocularis*, conventional and acid blotting produced essentially complementary results with regard to the molecular mass of recognized glycoproteins. Following conventional blotting, ConA, SBA, WGA, PNA, and Jacalin produced distinct staining of glycoproteins, ranging from around 40 to >200 kDa; staining was mostly abolished by addition of the corresponding inhibitory sugars. Upon acid blotting, only the higher molecular mass components were labeled. For the laminated layer fraction of *E. vogeli*, the situation was different. Upon conventional blotting, SBA, WGA, and PNA specifically stained a smear of glycoproteins ranging from 60 to >200 kDa, whereas ConA exhibited similar labeling plus a marked staining of 2 bands of approximately 55 and 80 kDa. In contrast to lectin fluorescence, no Jacalin binding sites were detected on western blots of *E. vogeli* fractions (Fig. 32). Following acidic blotting, no staining of any glycoproteins could be seen with any of the lectins.



FIGURES 7–12. Comparison of the laminated layer of *E. vogeli* and *E. multilocularis*. *G* = germinal layer; *T* = tegument; *LL* = laminated layer. **7.** SEM of vesicle wall of *E. vogeli* metacestode. Bar = 70 μm . **8.** SEM of vesicle wall of *E. multilocularis* metacestode. Note the differences in the thickness of the laminated layer as indicated by arrowheads, as well as differences in texture. Bar = 100 μm . **9.** TEM of *E. vogeli* vesicle wall, fixed with tannic acid. Bar = 2 μm . **10.** Higher magnification view of *E. vogeli* vesicle wall, showing microtriches (MT) protruding into the laminated layer. Bar = 0.57 μm . **11.** Section through the vesicle wall of *E. multilocularis*, fixed with tannic acid. Bar = 2.7 μm . **12.** Higher magnification view of *E. multilocularis* vesicle wall. Note the ultrastructural differences between *E. vogeli* and *E. multilocularis*. Bar = 0.5 μm .



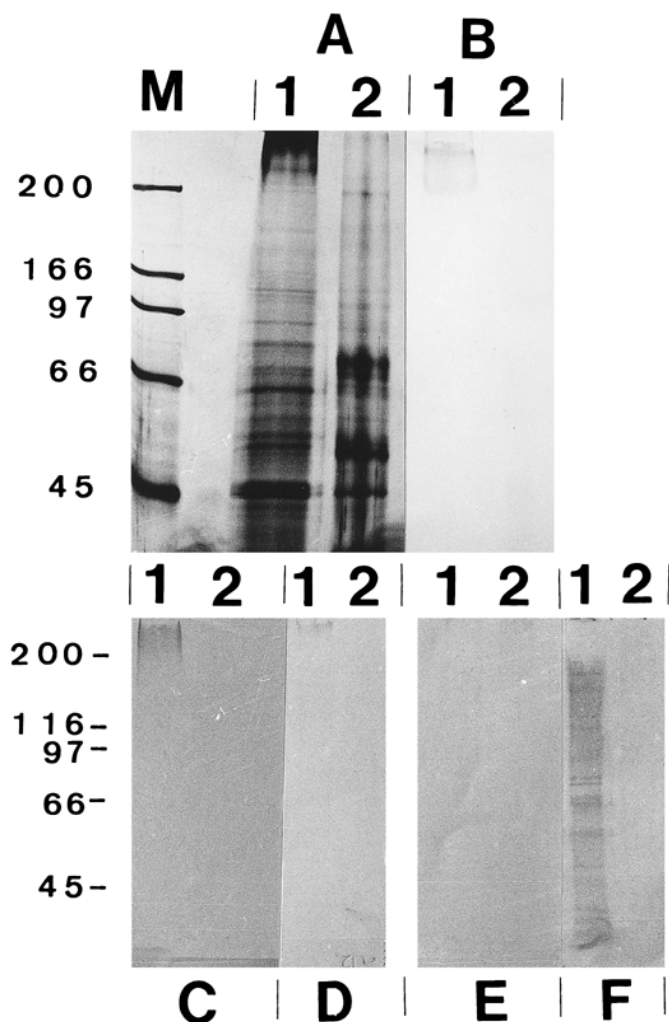


FIGURE 23. A. Silver stained SDS-PAGE of purified *E. multilocularis* (lane 1) and *E. vogeli* (lane 2) laminated layer. B. Corresponding PAS staining, revealing the high-molecular-mass glycans in the *E. multilocularis* laminated layer fraction only (lane 1). C, D. Corresponding western blot transferred using acid blotting buffer. E, F. Corresponding western blot transferred using conventional blotting buffer. C and E are labelled using a commercially available carbohydrate detection kit. D and F are stained with the polyclonal anti-*E. multilocularis* antiserum.

DISCUSSION

There is substantial evidence for the crucial role of the metacystode laminated layer in protection of the parasite from host defense mechanisms (Gottstein and Hemphill, 1997). However, there is still a lack of knowledge of the comparative biology of this laminated layer in view of its direct host-parasite interplay. In the present study, several main characteristics of the laminated layer of *E. vogeli* and *E. multilocularis* metacystodes were compared. These studies were carried out using parasite mate-

rial that had been cultured in vitro for several months. We thus created directly comparative conditions, as opposed to the putative use of in vivo generated parasites, which could have been affected by the incorporation of host-related features into the structures under study.

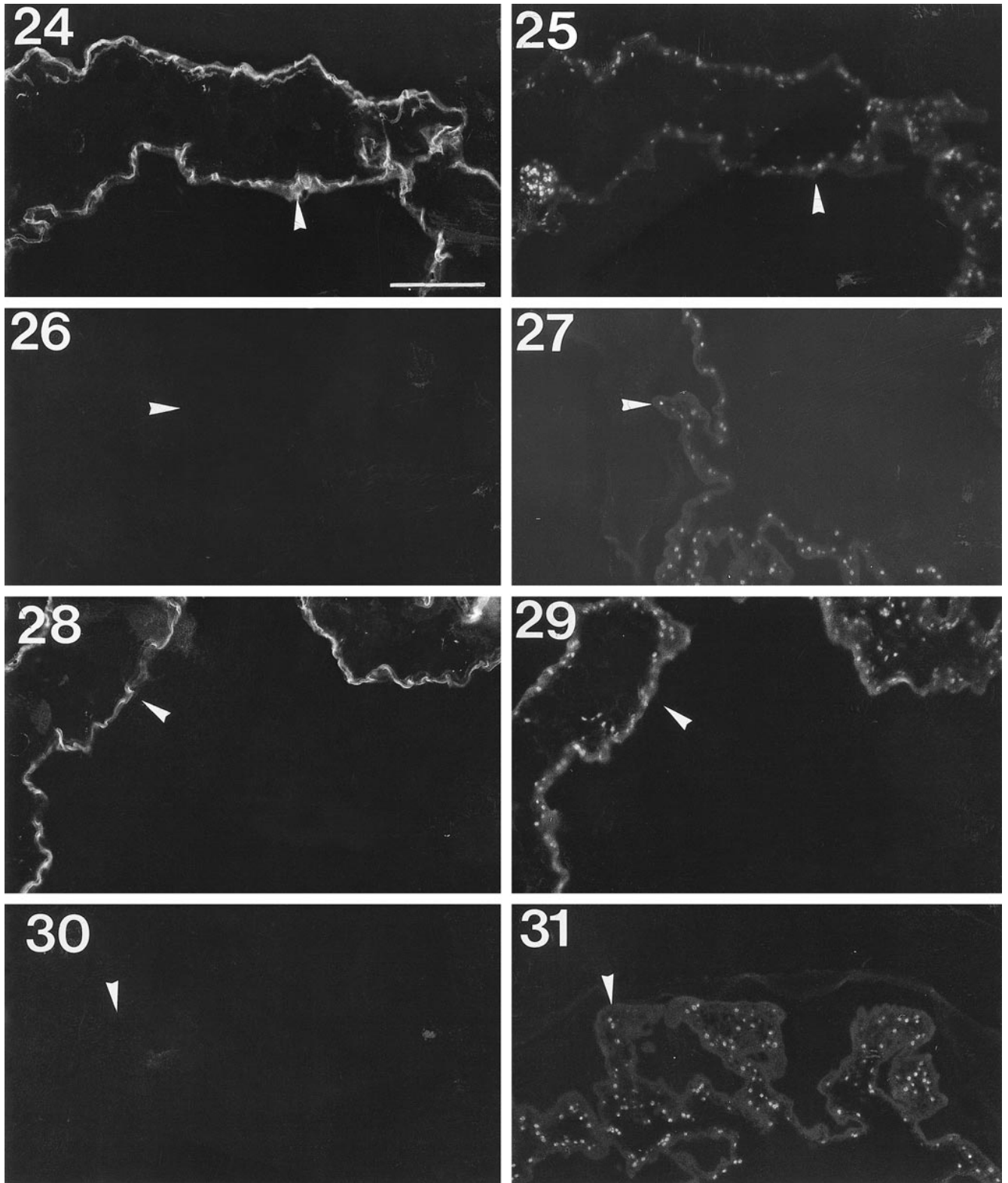
When metacystodes from *E. multilocularis* and *E. vogeli* were isolated from the peritoneal cavity of infected C57BL/6 mice, distinct differences were evident with respect to morphological and growth characteristics. Metacystodes of *E. multilocularis* were found mostly attached to or integrated within adjacent host tissues and often were encapsulated within host connective tissue. In contrast, attachment of *E. vogeli* metacystodes to serosal surfaces within the peritoneum was less evident. Rather, they were growing either individually or in small groups but were not tightly associated with host tissue, similar to the observations recently provided by Rausch and D'Alessandro (1999). Both parasites also showed clear differences with regard to in vitro culture. Whereas *E. multilocularis* exhibited a marked exogenous proliferation accompanied by a continuous increase in size and late protoscolex development (Hemphill and Gottstein, 1995), *E. vogeli* did not show a similar proliferation but rather a much more pronounced progression of protoscolex formation. These protoscolexes were mostly found within brood capsules, or they developed directly from the germinal layer (see Figs 1–6). In *E. multilocularis*, in vitro protoscolex development appeared to take place within brood capsules. We do not know whether these differences reflect inherent biological properties of the 2 species or whether they were influenced by the conditions of in vitro culture.

The marked differences with regard to exogenous proliferation of the metacystodes could be related to the properties of the laminated layer, which is considerably thicker in *E. vogeli* than in *E. multilocularis*. Both SEM and TEM demonstrated differences between the 2 parasites in both morphology and ultrastructure (see Figs. 7–12). The ultrastructure of the metacystode has been extensively investigated in *E. multilocularis* (Mehlhorn et al., 1983; Delabre et al., 1987; Hemphill and Gottstein, 1995; Ingold et al., 1999). It consists of an inner germinal layer and, by the tegument, a syncytial layer that surrounds the entire metacystode. Whereas we could not detect any major ultrastructural differences between *E. multilocularis* and *E. vogeli* metacystode tissue, the situation was different with regard to the laminated layer. Besides the difference in thickness, there is a marked discrepancy with regard to structural organization in the 2 species, as revealed after tannic acid fixation. This finding suggested that there could also be marked compositional dissimilarities in the laminated layer between *E. multilocularis* and *E. vogeli*.

Our investigations appear to confirm these findings. The carbohydrate composition of the *E. vogeli* laminated layer was analyzed using either lectin fluorescence microscopy (Figs. 13–22) or lectin blotting (Fig. 23). Although clear differences between *E. vogeli* and *E. multilocularis* could be observed, the

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FIGURES 13–22. Lectin fluorescence microscopy of *E. vogeli* metacystodes embedded in LR-White resin. Sections were stained with biotinylated lectins followed by incubation in streptavidin-FITC. 13. Labeling with ConA. 14. Labeling with ConA and Hoechst 33258 to indicate the parasite nuclei and the border between laminated and germinal layer (indicated by arrowheads). 15. Labeling with SBA. 16. Labeling with SBA and Hoechst 33258. 17. Labeling with WGA. 18. Labeling with WGA and Hoechst 33258. 19. Labeling with PNA. 20. Labeling with PNA and Hoechst 33258. 21. Labeling with Jacalin. 21. Labeling with Jacalin and Hoechst 33258.



FIGURES 24–31. Immunofluorescence staining of LR-White-embedded *E. multilocularis* (24, 28) and *E. vogeli* (26, 30) metacystodes, labeled with a polyclonal antiserum directed against the laminated layer of *E. multilocularis* metacystodes (24, 26) or with the mAb G11 directed against the *E. multilocularis* Em2 carbohydrate antigen (28, 30). Note the absence of immunostaining from *E. vogeli* metacystodes. 25, 27, 29, 31. Corresponding sections stained with Hoechst 33258 to indicate the location of the parasite nuclei.

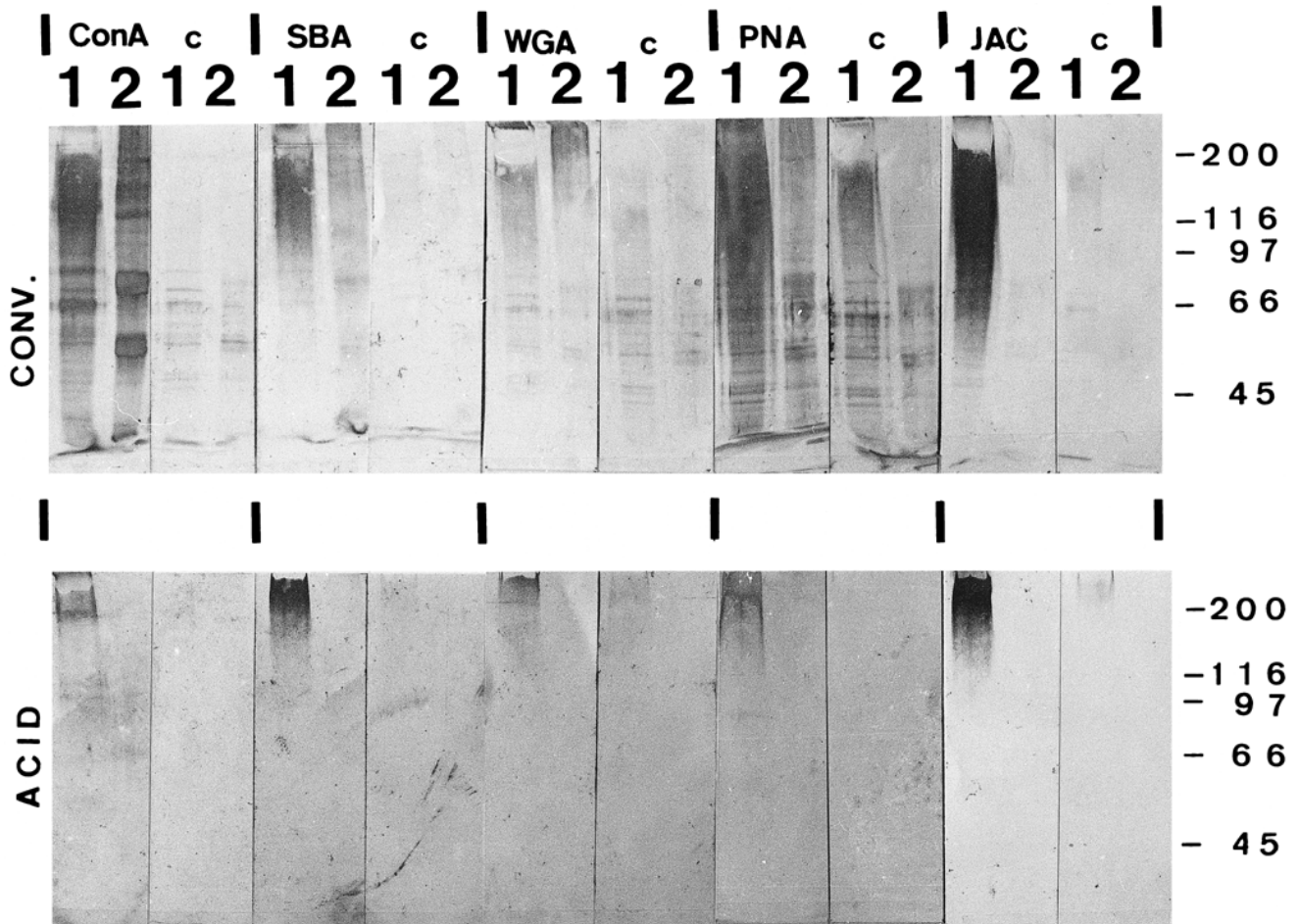


FIGURE 32. Comparative lectin blotting of purified laminated layer fractions from *E. multilocularis* (lane 1) and *E. vogeli* (lane 2), with transfer to nitrocellulose undertaken by conventional blotting (upper) and acid blotting (lower). Lectin staining and corresponding inhibition controls are shown. Note the absence of any lectin staining in the *E. vogeli* fractions under acid blotting conditions.

significance of the results obtained through the use of lectins should be treated with caution for the following reasons. First, it has been shown that for a given protein or peptide, processing of its oligosaccharides might vary in different or even closely related cell types (Sheares and Robbins, 1986), and for a given glycoprotein a spectrum of different structures can be obtained (Hubbard and Ivatt, 1981). This means that only a small population of a given glycoprotein could be recognized by a single lectin. Second, lectins do not bind only to terminal nonreducing sugars in glycoconjugates; they may also bind mono- and oligosaccharides in internal positions in a manner that is largely conformation dependent. Third, the capacity of ConA to bind to a given glycan can be completely prevented by the addition of terminal carbohydrate residues, because of steric hindrance (Griffiths, 1993). Thus, based on lectin binding, it is difficult to make definitive conclusions regarding the final structure and composition of oligosaccharides. However, the laminated layer of *E. vogeli* is clearly devoid of those high-molecular-mass glycans that have been recently shown to constitute major structural elements of the laminated layer of *E. multilocularis* metacystodes (Ingold et al., 2000). As indicated by silver staining, there are also clear differences with regard to the protein composition in the laminated layer of the 2 species, and the lack of

cross-reactivity using polyclonal antisera and monoclonal antibodies demonstrates the intrinsic differences in antigenicity (Figs. 24–31).

Nevertheless, it is still most likely that the laminated layers in *E. vogeli* and *E. multilocularis* are at least functionally similar. For *E. multilocularis*, there are several indications that the laminated layer may play an important role in protecting the developing metacystode from host immune reactions. Screening of human patients has shown that the laminated layer can remain within the infected host tissue for a long time, even after spontaneous death of the larval parasite (Rausch et al., 1987; Condon et al., 1988; Lanier et al., 1988). Deplazes and Gottstein (1991) showed that the laminated layer-associated parasite carbohydrate antigen Em2 was primarily expressed in oncospheres, which initiated synthesis of the laminated layer within 2 wk of hatching in vitro, the same period of time required for a host to generate a specific systemic immune response. In a murine animal model, no protection is achieved against established metacystodes already carrying the laminated layer, whereas protoscoleces lacking the laminated layer and the Em2 antigen cannot induce secondary alveolar echinococcosis in rodents (Gottstein et al., 1992). Immunogold electron microscopy 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 and Hemphill, 1997).

Similar information with regard to *E. vogeli* remains to be obtained. Because we have now established a procedure for cultivating *E. vogeli* metacystodes in vitro under conditions similar to those for *E. multilocularis*, we should be able to perform further comparative studies directed at elucidating the mechanisms that enable *E. multilocularis* and *E. vogeli* metacystodes to establish themselves within the host. Because the heavily glycosylated laminated layer represents the outermost surface of these parasites, many answers could be obtained by a more detailed molecular analysis of the laminated layer and its associated molecules, followed by studies on the specific interactions between these components and defined host immune and nonimmune cells.

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