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Echinococcus multilocularis Alkaline Phosphatase as a Marker for Metacestode Damage Induced by In Vitro Drug Treatment with Albendazole Sulfoxide and Albendazole Sulfone

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Alveolar echinococcosis (AE) is caused by the metacestode stage of the fox tapeworm *Echinococcus multilocularis*. The disease affects the human liver and occasionally other organs and is fatal if treatment is unsuccessful. The present chemotherapy of AE is based on the administration of benzimidazole carbamate derivatives, such as mebendazole and albendazole. Albendazole treatment has been found to be ineffective in some cases, parasitostatic rather than parasiticidal, and the recurrence rate is rather high. Therefore, chemotherapy usually involves the lifelong uptake of massive doses of albendazole and new treatment options are urgently needed. In order to avoid costly and time-consuming animal experimentation, a first step in searching for novel parasiticidal compounds could be the in vitro drug screening of novel compounds by employing metacestode cultivation. However, presently used techniques (e.g., transmission electron microscopy) for determination of parasite viability involve costly equipment and time-consuming preparation of rather large amounts of parasite material. We therefore searched for a parasite marker which can be easily traced and the presence or absence of which is indicative of parasite viability. In this study we show that the increase of *E. multilocularis* alkaline phosphatase activity in culture supernatants during in vitro drug treatment with albendazole derivatives correlates with the progressive degeneration and destruction of the metacestode tissue. The inexpensive and rapid assay presented here will serve as an ideal tool for performing first-round in vitro tests on the efficacy of a large number of antiparasitic compounds.

Alveolar echinococcosis (AE) is prevalent in many areas of the Northern Hemisphere. In regions where it is endemic, such as Alaska, Central Europe, and Japan, it is well known as a public health hazard to humans (7, 22). The disease, caused by the metacestode (larval) stage of *Echinococcus multilocularis*, is one of the most lethal helminthic infections of humans. The adult tapeworm exists as an enteric parasite in the fox and in a few other carnivores, such as wolf, cat, and dog. Eggs which are accidentally ingested by the intermediate host are the source of infections in humans. The egg releases an oncosphere, which, upon hatching, penetrates the intestinal mucosa and enters the circulation. The oncosphere is transported primarily to the liver, where it develops into a vesiculated, tumorlike metacestode tissue. Metacestodes may develop secondarily in the lung, brain, and other organs of the affected intermediate host, where voluminous lesions will inflict organ dysfunction, often leading to death (1).

Treatment of AE requires surgical intervention, if possible radical, combined with chemotherapy using benzimidazole carbamate derivatives, such as albendazole and mebendazole (2, 3). Chemotherapy has been shown to exert a parasitostatic rather than a parasiticidal effect. A further disadvantage of the present treatment is that it has, in certain cases, proven to be ineffective, and the recurrence rate is rather high once chemotherapy is stopped (4). Thus, the development of new treatments of AE is anticipated. New antihelminthic compounds, such as paraherquamides, cyclic depsipeptides, nitazoxanides, and others, may be interesting candidates (8).

Traditionally, assessment of the efficacy of drugs against *E. multilocularis* metacestodes has always relied on animal experimentation using mice or gerbils (6, 23, 24, 27, 28). Such investigations are usually associated with high costs and time-consuming analyses. More recently, in vitro culture models have been introduced which allowed scientists to grow parasite larvae from infected tissue under standardized conditions but did still preserve the infectivity of the resulting metacestodes (9, 16, 17). Researchers have previously reported on the in vitro culture of *E. multilocularis* metacestodes (10, 12, 13, 14) and have shown by maintaining parasites in the presence of the albendazole derivatives albendazole sulfoxide (ABZSO) and albendazole sulfone (ABZSN) and evaluating their efficacy that this model could be used for primary drug screening assays and studies on the mode of action and the targeting of drugs (13). Methods included measurement of drug uptake in the vesicle fluid by high-performance liquid chromatography (HPLC), assessment of metabolic changes within the vesicle fluid by nuclear magnetic resonance (NMR) spectroscopy, and evaluation of the damage imposed on the parasite by these components using transmission electron microscopy (TEM) (13). However, all these procedures require either a relatively large amount of parasite material or are, due to their complexity, rather time-consuming. In addition, only a small portion of the parasite tissue can be studied by TEM. Thus, these tech-

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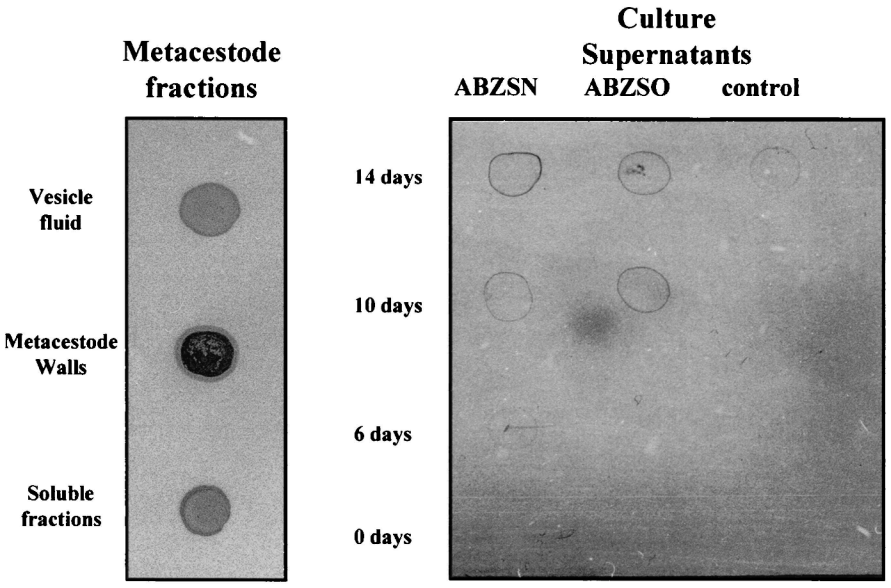

FIG. 1. Demonstration of EmAP activity as revealed by dot blotting in *E. multilocularis* vesicle fluid, insoluble metacestode walls, soluble metacestode tissue components, and medium supernatants after different time points of in vitro culture in the presence and absence of 10 μg/ml of ABZSO or ABZSN. Number of days of in vitro treatment is indicated.

niques are not applicable in mass screenings for chemotherapeutically interesting compounds. We have therefore searched for a biochemical marker which could be indicative of parasite damage or death and which could be easily traced following in vitro culture and drug treatment of metacestodes.

We show here that the *E. multilocularis* alkaline phosphatase (EmAP) (25, 26) can be used as such a marker. Following in vitro drug treatment, the concentration of EmAP is dramatically increased in medium supernatants of drug-treated parasites compared to those of nontreated larvae and its activity is easily detected in a standard colorimetric assay using *p*-nitrophenyl phosphate as a substrate. The increase in EmAP concentrations in the medium supernatant was accompanied by significant damage of the germinal layer-associated tissue, as visualized by scanning electron microscopy (SEM). In addition, TEM analysis showed that increasing levels of EmAP activity in culture supernatants could also be correlated to ultrastructural alterations occurring on the most outer, acellular, and carbohydrate-rich laminated layer of the parasite.

MATERIALS AND METHODS

Experimental design. In this study we correlated the activity of EmAP in culture medium supernatants of metacestodes following cultivation in the presence and absence of the albendazole derivatives ABZSO and ABZSN with the damage and ultrastructural alterations induced by these drugs as assessed by SEM and TEM. The following experimental approach was used: (i) in vitro cultivation of blocks of tissue infected by *E. multilocularis* and isolation of individual, free-floating metacestodes; (ii) incubation of metacestodes in medium containing defined amounts of ABZSO and ABZSN; (iii) harvesting of medium supernatants at defined time points and assessment of EmAP activity; (iv) SEM and TEM analysis of control and drug-treated parasites; and (v) immunolocalization of EmAP in control and drug-treated parasites by immunogold TEM.

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

In vitro cultivation of parasites. In vitro cultivation of *E. multilocularis* metacestodes was carried out as described previously (9). Briefly, gerbils (*Meriones unguiculatus*) were infected intraperitoneally with the *E. multilocularis* clone KF5 and the isolate IM280. After 1 to 2 months, the animals were euthanized, and the parasite tissue was recovered from the peritoneal cavity under aseptic conditions. The tissue pieces were cut into small tissue blocks (volume, 0.5 cm^3), which were washed twice in Hanks balanced salt solution. Two pieces of tissue were placed in 40 ml of culture medium (RPMI 1640 containing 12 mM HEPES, 10% fetal calf serum ([FCS], 2 mM glutamine, 200 U of penicillin/ml, 200 μg of streptomycin/ml, and 0.50 μg of amphotericin B/ml) to generate free-floating vesicles (see below). Tissue blocks were kept in tightly closed culture flasks (75 cm^2) placed in upright position in an incubator at 37°C, 5% CO_2, with medium changes every 2 to 4 days.

Drug treatments and recovery of medium supernatants, vesicle fluid, and metacestode tissue. Intact vesicles with diameters of 1 to 5 mm were harvested after 3 to 4 weeks of tissue block cultivation. The time of vesicle collection was selected in order to obtain actively growing and proliferating parasites. The metacestodes were pooled, washed three times in sterile water, and divided again into separate cultures with approximately 50 vesicles in 15 ml of RPMI 1640 medium containing 2 mM glutamine, 200 U of penicillin/ml, 200 μg of streptomycin/ml, and 0.50 μg of amphotericin B/ml but completely lacking FCS and phenol red. ABZSO and ABZSN (kindly provided by R. J. Horten, SmithKline Beecham, London, United Kingdom) were prepared as stock solutions of 10 mg/ml in dimethyl sulfoxide (DMSO). These reagents were added to the cultures at a 1:1,000 dilution, yielding a final concentration of 10 μg/ml. For each experiment, the appropriate controls included (i) a culture containing the equal amount of DMSO and (ii) a culture in RPMI medium alone. The parasites were incubated at 37°C and 5% CO_2. After defined time points as indicated for Fig. 1, 300 μl of culture supernatant was collected and centrifuged at 10,000 × *g* for 30 min at 4°C and the supernatant was recovered and stored at −80°C before further use.

Separation of vesicle fluid and parasite tissue was carried out as described (12). Briefly, in vitro-cultured metacestodes were washed twice in distilled water. The water was carefully aspirated, and the tube containing the vesicles was placed on ice. The metacestodes were then gently broken up by using a pipette, and the preparation was centrifuged at 3,000 × *g* for 30 min at 4°C. The supernatant (containing vesicle fluid) was collected and spun again for 30 min at 10,000 × *g* at 4°C and was stored at −80°C before further use. The pellet containing vesicle wall tissue was either processed for TEM (see below) or was resuspended in phosphate-buffered saline (PBS) and also stored at −80°C before further use.

Determination of EmAP activity. Qualitative measurement of EmAP activity was performed by dot blot analysis: 20 μl of each culture supernatant, as well as 10 μl of vesicle fluid and of metacestode extract, was spotted onto a nitrocellulose filter, and the filter was air dried at room temperature for 1 h. Subsequently, the filter was rehydrated in PBS and was incubated in alkaline phosphatase

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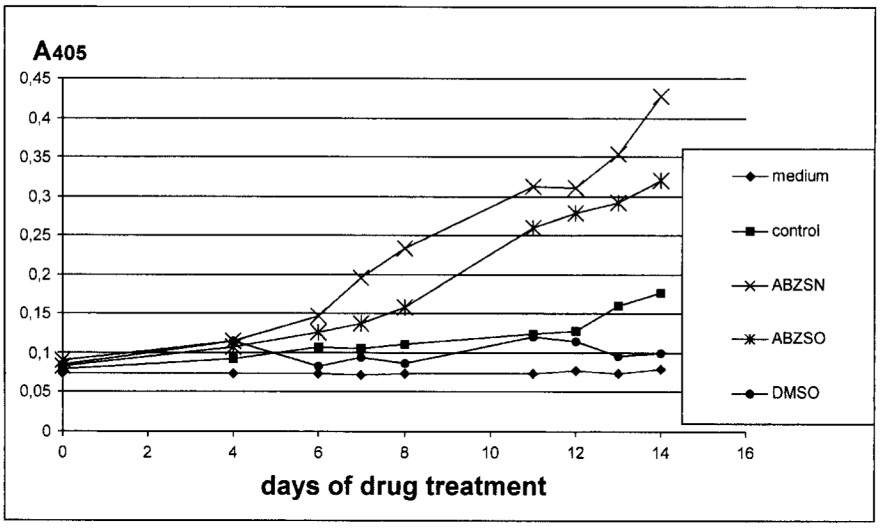

FIG. 2. Time course experiment demonstrating elevated EmAP activity in culture supernatants of *E. multilocularis* metacestodes treated with 10 μg of ABZSO or ABZSN per ml. Controls include RPMI 1640 medium alone (without parasites; designated medium), RPMI 1640 medium plus metacestodes (control), or RPMI 1640 medium plus metacestodes plus the corresponding amount of DMSO (DMSO). Note the increase of EmAP levels starting at day 4 in drug-treated cultures.

reaction buffer (100 mM Tris, 100 mM NaCl, and 10 mM $MgCl_2$, pH 9.5) containing 4-nitrotetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (12). The reaction was allowed to proceed for 2 to 3 min; subsequently, the filter was washed in distilled water and air dried.

For quantitative assessment of alkaline phosphatase activity, 30 μl from each culture supernatant was mixed with 170 μl of alkaline phosphatase enzyme-linked immunosorbent assay (ELISA) substrate buffer (0.5 M ethanolamine and 0.5 mM $MgCl_2$, pH 9.8) containing *p*-nitrophenyl phosphate (1 mg/ml). Two hundred microliters of each sample was pipetted into wells of a 96-well ELISA plate, and the plate contents were incubated for 30 min at 37°C. A_{405} values were read on a Dynatech MR7000 ELISA reader.

SEM. In vitro-cultured metacestodes were processed for SEM analysis as described (15). Briefly, freshly isolated vesicles were fixed in 2.5% glutaraldehyde in 100 mM phosphate buffer for 4 h at room temperature, followed by postfixation in 2% OsO_4 in phosphate buffer. Samples were extensively washed in distilled water and dehydrated in acetone and were sublimation dried in Peldri II (Plano GmbH, Marburg, Germany) as described previously (11). Specimens were placed onto glass coverslips, sputter coated with gold, and inspected on a JEOL 840 scanning electron microscope operating at 25 kV.

TEM. Freshly isolated vesicle walls were processed for TEM as described (15). Briefly, they were fixed for 4 h at room temperature in 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.2, containing 0.5% of tannic acid, followed by postfixation in 2% OsO_4 in phosphate buffer. Samples were extensively washed in distilled water and were incubated in 1% uranyl acetate for 1 h at 4°C, followed by several washes in buffer. They were dehydrated in a graded series of ethanol and were subsequently embedded in Epon 812 resin according to the method described by Hemphill and Croft (11). Polymerization of the resin was carried out at 65°C overnight. Sections were cut on a Reichert and Jung ultramicrotome and were loaded onto 300-mesh copper grids (Plano GmbH). Staining with uranyl acetate and lead citrate was performed as described previously (11).

Immunogold labeling TEM. In vitro drug-treated or untreated *E. multilocularis* metacestodes were fixed in 3% paraformaldehyde–0.05% glutaraldehyde in 100 mM phosphate buffer for 1 h on ice. They were then washed extensively in PBS and were incubated in PBS–50 mM glycine for 1 h on ice, followed by sequential dehydration in a graded series of ethanol at −15°C for 5 min each. Embedding in LR-White resin (Sigma) was carried out at −15°C, with three changes of fresh resin every 24 h. Polymerization of the resin was achieved at 55°C over a time span of 24 h. Ultrathin sections were loaded onto Formvar carbon-coated 200-mesh Nickel grids (Plano) and were stored not longer than 48 h at 4°C prior to use. The following steps were performed at room temperature. Blocking of unspecific binding sites was done in PBS–1% bovine serum albumin for 2 h, followed by incubation with an affinity-purified anti-EmAP antibody diluted 1:1 in blocking buffer (20). Following washing in PBS, grids were incubated with a goat anti-rabbit immunoglobulin G antibody conjugated to 10-nm gold particles (Amersham, Zürich, Switzerland) for 1 h. Subsequently, they were washed extensively in PBS, were air dried, and were stained with uranyl acetate and lead citrate (11).

RESULTS

Increase of EmAP activity in culture supernatants of drug-treated *E. multilocularis* metacestodes. As we were searching for a molecule which could be used for an easy and rapid assessment of impairment of parasite viability during in vitro drug testing, we investigated EmAP activity in culture supernatants at different time points following the addition of either ABZSO or ABZSN into the culture medium. In vitro drug treatment assays had to be performed in the absence of FCS in the medium, as the serum-derived alkaline phosphatase activity was producing intense background (data not shown). Preliminary experiments had shown that both isolated vesicle fluid as well as vesicle tissue exhibited EmAP activity, as evidenced by dot blot analysis on nitrocellulose filters (Fig. 1). Time course experiments (as shown in Fig. 2) demonstrated that after 8 to 10 days, the EmAP activity in culture supernatants of drug-treated parasites was dramatically enhanced compared to that in corresponding supernatants of control cultures. After 12 to 14 days of in vitro culture, a rise in EmAP activity was also observed in supernatants of control cultures, albeit to a much lower extent. These experiments were repeated six times, and all provided essentially identical results (Fig. 2) and were confirmed by dot blot analysis (Fig. 1).

SEM. In order to correlate this dramatic increase of EmAP activity in culture supernatants of drug-treated parasites with parasite viability or nonviability, both control and drug-treated parasites were examined by SEM. SEM analysis showed that nontreated metacestodes exhibited a largely intact germinal layer composed of a multitude of different cell types (Fig. 3A and B). Only few cells with impaired morphology could be seen. The morphological features of parasites after 10 days of

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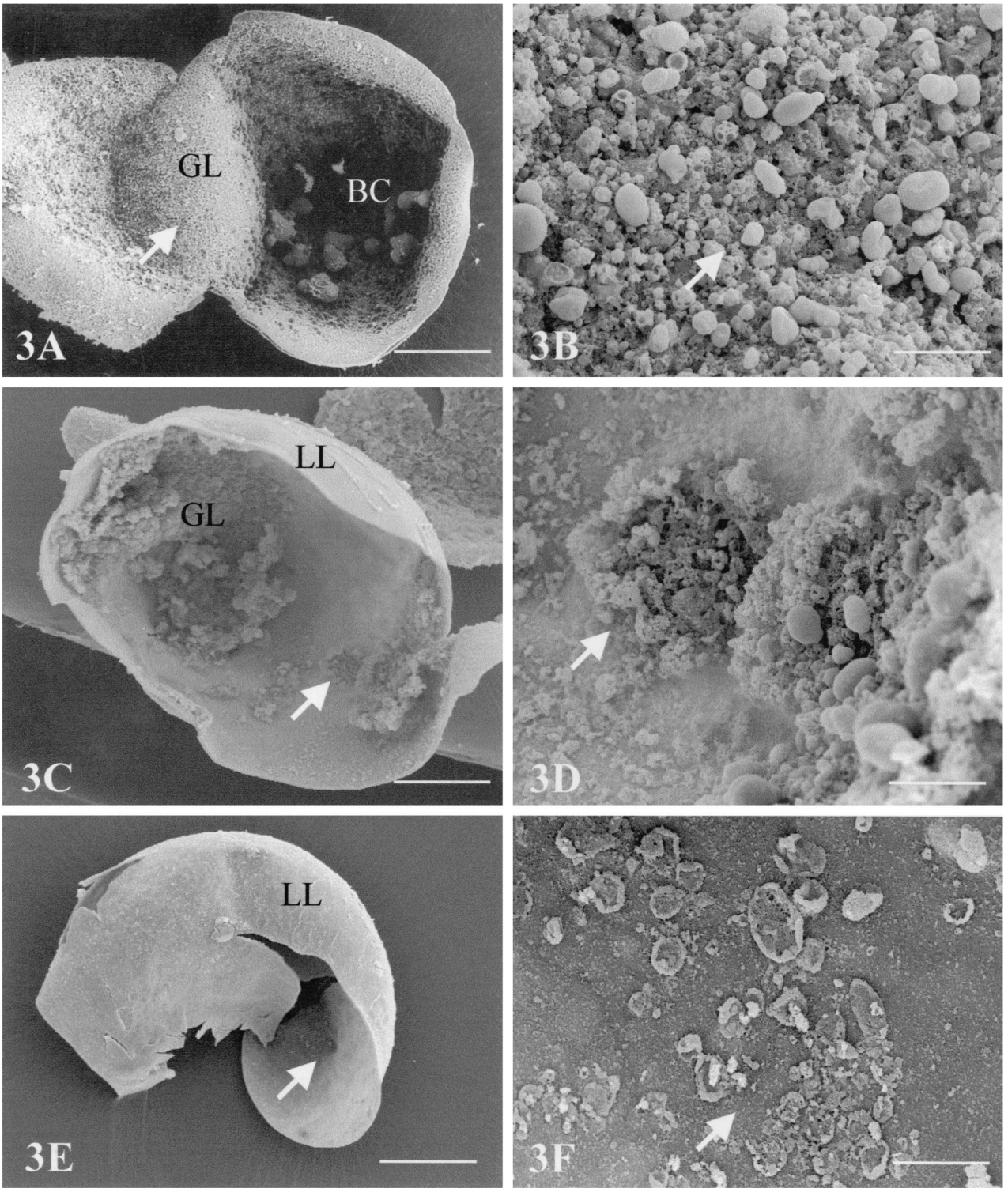

FIG. 3. SEM nontreated (A and B) or ABZSO-treated (C to F) *E. multilocularis* metacestodes. GL, germinal layer; LL, laminated layer. (A and B) Control metacestodes cultured in vitro in the presence of DMSO (1:1,000) but in the absence of any drugs. Note that most cells exhibit an intact morphology. Bar = 1.2 mm (A) or 200 μm (B). (C to F) SEM of metacestodes cultured in vitro in the presence of ABZSO for 10 days (C and D) and of ABZSN for 14 days (E and F). (C) Large portions of the germinal layer have disintegrated after 10 days of drug treatment and are detached from the laminated layer (bar = 900 μm). (D) Higher-magnification view of image in panel A (bar = 200 μm). (E) After 14 days, only metacestode "ghosts," comprised of the acellular laminated layer, are found (bar = 1.2 mm). (F) Higher-magnification view onto the interior surface of the laminated layer. Note the presence of largely destroyed cells (bar = 180 μm).

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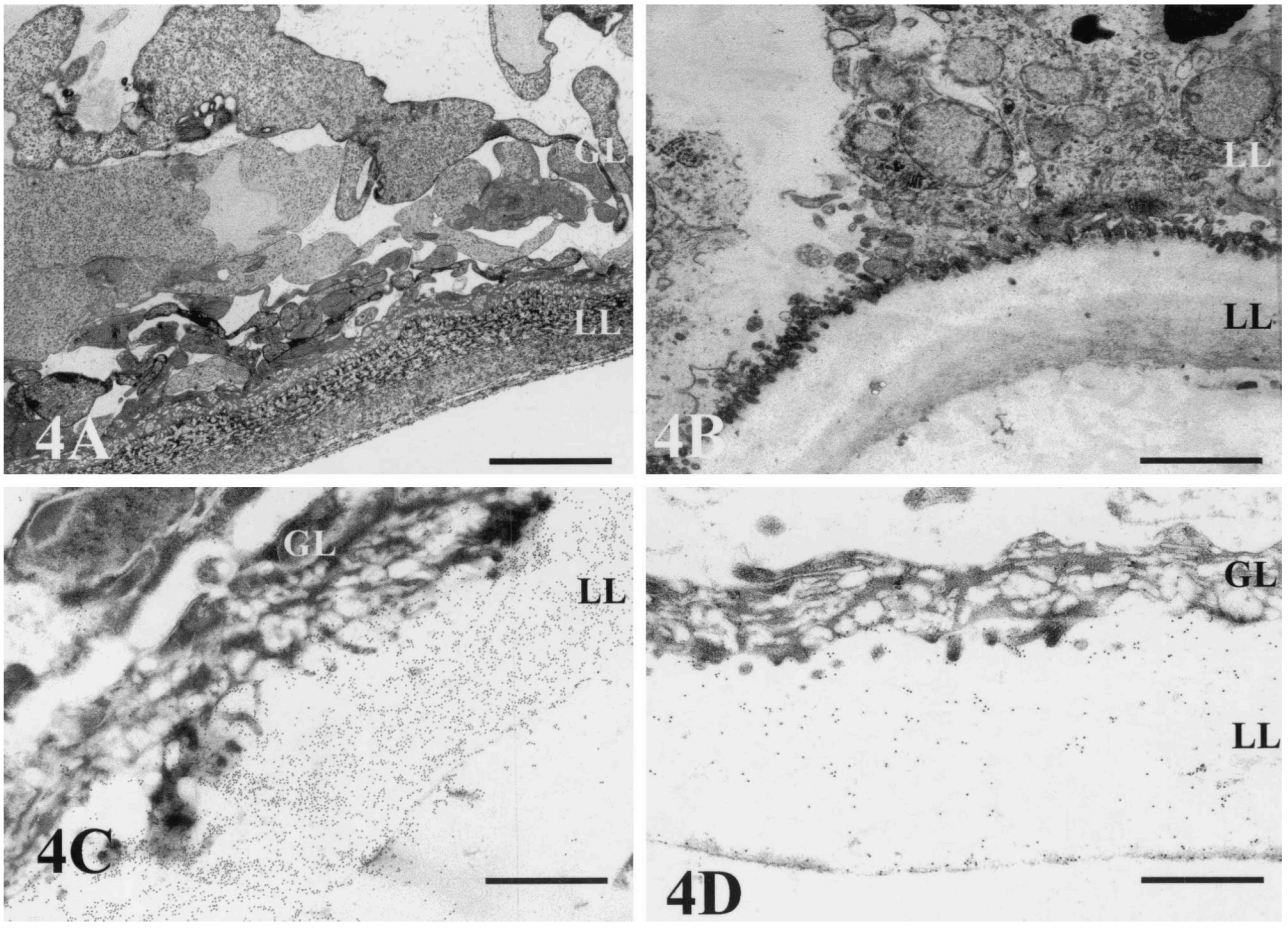

FIG. 4. Comparative assessment of ultrastructure (A and B; bars = 4 and 3 μm, respectively) and EmAP immunogold labeling (C and D; bars = 2.3 and 1.9 μm, respectively) in control (A and C) versus ABZSO-treated (B and D) metacestodes 10 days after initiation of drug treatment. Note the lack of the typical microfibrillar pattern in the matrix of control metacestodes (A) compared to the amorphous appearance of the laminated layer (LL) in drug-treated parasites (B). The density of EmAP immunogold labeling within the laminated layer in drug-treated parasites (D) is significantly lower than in control metacestodes (C). GL, germinal layer.

in vitro ABZSO treatment as investigated by SEM are shown in Fig. 3C and D: in many areas of the metacestode, the germinal layer was largely disintegrated and only a fraction of the parasite tissue appeared to be still attached to the interior surface of the morphologically still intact, acellular laminated layer. At 14 days of in vitro drug treatment, mostly metacestode "ghosts," composed exclusively of the acellular laminated layer, were found (Fig. 3E and F). Closer inspection of the inner surface of such ghosts revealed the presence of only cellular residues of the germinal parasite tissue (Fig. 3F). Essentially identical results were obtained when parasites treated with ABZSN were investigated (data not shown). Thus, the increase in EmAP activity in medium supernatants following in vitro drug treatment did largely correlate with impaired parasite viability and cellular destruction.

TEM. A detailed TEM analysis of the ultrastructural alterations of the germinal layer-associated tissue imposed upon in vitro drug treatment of *E. multilocularis* metacestodes has been previously performed (13). However, in this study we also observed distinct differences with regard to the structural appearance of the most outer laminated layer in drug-treated versus control metacestodes. We could see ultrastructural differences within the matrix of the laminated layer when comparing drug-treated and untreated parasites. In untreated parasites, the laminated layer displayed a characteristic microfibrillar pattern (Fig. 4A), while this distinct microfibrillar pattern was largely missing in the laminated layer of drug-treated metacestodes (Fig. 4B). Thus, the increase of EmAP activity in the culture supernatants is paralleled by a progressive loss of the distinct, largely carbohydrate-based, ultrastructural characteristics of the laminated layer.

EmAP has been previously found to be a major component of the laminated layer of *E. multilocularis* metacestodes (20). In control cultures, fixed and processed for immunogold labeling following 14 days of in vitro culture, we could indeed localize this protein almost exclusively within the laminated layer, as evidenced by immunogold labeling employing anti-EmAP antibodies (Fig. 4C). Only marginal staining could be observed within the germinal layer tissue. In contrast, immunogold labeling of ABZSO- and ABZSN-treated metacestodes showed that the anti-EmAP labeling intensity within the laminated layer was dramatically diminished (Fig. 4D). Thus, the increase in EmAP activity in the culture supernatants is ac-

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companied by the loss of EmAP immunoreactivity within the laminated layer.

DISCUSSION

Previous studies (13) have established that in vitro drug treatment of *E. multilocularis* metacestodes could represent a valuable alternative to the animal experimentation practiced to date, as it allows one to monitor drug uptake by HPLC, to study by NMR metabolic alterations induced through drug treatment, and to investigate by TEM ultrastructural changes imposed through drugs (13). However, when it comes to performing drug-testing assays with a multitude of chemotherapeutically interesting reagents, these techniques suffer from their complexity or require large amounts of parasite material. TEM is helpful but time-consuming, and only a small portion of the metacestode can be investigated using the electron microscope. Thus, our aim was to set up an assay for investigating parasite viability which would be more practical and easier to perform. This required the identification of a parasite marker which would be indicative for impaired parasite viability in vitro and which would be relatively easy to monitor.

E. multilocularis metacestodes possess a high alkaline phosphatase (EC 3.1.3.1) activity which has been previously purified and characterized (19, 25). The parasite enzyme was found to exhibit unique properties compared to the corresponding enzyme of mammalian tissues, as its activity was 50-fold higher than that of the alkaline phosphatase from gerbil and sheep liver tissue. Other features, such as resistance towards heat denaturation, differences in response to various alkaline phosphatase inhibitors, and slight differences in molecular weight and isoelectric point suggested that EmAP could be intrinsically different from its mammalian counterparts (19, 25). Previous investigations had demonstrated that EmAP is highly abundant in those parasite compartments crucially involved in interacting with the host, most notably on the outer laminated layer of *E. multilocularis* metacestodes and on the periphery of protoscoleces (20). Due to its abundance at the host-parasite interface and its high activity, it is conceivable that EmAP represents a molecule of considerable importance for this parasite, as it may be involved in the acquisition of nutrients (5, 21) as well as in the modulation of phosphorylation-dependent events at the host-parasite boundary: for instance, those interactions initiated by host-effector cells. In addition, due to antibody cross-reactivity and similar localization, it was suggested that EmAP and the major laminated layer-associated carbohydrate antigen Em2 were antigenically related (20).

It was previously shown that the serological response of patients against EmAP could reflect parasite viability following surgery and/or chemotherapy (26). For instance, antibodies directed against EmAP were detected in patients who were suffering from AE which had been treated by surgery and/or chemotherapy but who then experienced a relapse. Thus, an increase in anti-EmAP antibody titers in those patients was predictive for a recurrence (26). However, in patients undergoing chemotherapy, the amount of anti-EmAP antibodies found in the corresponding sera was dependent on the type of treatment, most notably due to the differential mode of action of the chemotherapy agents used. A further observation was that at the time of initiation or reinitiation of albendazole or mebendazole treatment, an increase in anti-EmAP antibody titers was observed. This was interpretated to be an effect of EmAP release by the parasite (26).

In our study, in vitro-cultured metacestodes treated with ABZSO and ABZSN for up to 14 days released markedly higher EmAP activity into the culture supernatant than did control cultures. Release of EmAP into the medium was paralleled by progressive destruction and disintegration of the cellular organization of the metacestode germinal layer tissue, as visualized by SEM. In contrast, the overall morphology and cellular organization of the germinal layer were not, or only slightly, impaired during in vitro cultivation in the absence of the benzimidazole carbamate derivatives. Both the demonstration of EmAP activity by the use of *p*-nitrophenyl phosphate as a substrate and visualization of the morphological damage imposed upon the germinal layer-associated tissue by SEM represent techniques which consume far less time and material than do the previously demonstrated methods involving HPLC, NMR, and TEM (13). Thus, the assay introduced in this study allows a relatively easy and fast primary in vitro screening of a multitude of chemotherapeutically interesting agents.

The EmAP activity observed in culture supernatants could potentially originate from two distinct parasite compartments. First, as indicated in Fig. 1, isolated vesicle fluid itself exhibits EmAP activity. Our study shows that following in vitro drug treatment from day 7 onwards, EmAP activity in medium supernatants reaches increased levels in drug-treated parasite cultures compared to control cultures and that this corresponds approximately to the time point where the germinal layer of drug-treated metacestodes exhibits the most considerable ultrastructural damage (disappearance of microtriches, increasing degeneration of the germinal layer-associated tissue, and separation of the germinal and laminated layers), leading to irreversible destruction of the parasite (13). Thus, the loss of structural integrity is probably associated with the leakage of vesicle fluid, including EmAP activity, into the culture supernatant. Secondly, as evidenced by immunogold labeling using a previously characterized anti-EmAP antibody (20), EmAP is localized predominantly on the most outer, acellular, laminated layer of the parasite. In vitro drug treatment was accompanied by marked changes in the ultrastructural organization of the laminated layer, the matrix structure of which changed from microfibrillar to amorphous during drug treatment. This could be visualized by introducing tannic acid into the fixation protocol. In addition, the intensity of EmAP immunogold staining in the laminated layer was progressively diminished during the course of in vitro drug treatment. This indicates that impairment of parasite viability also affects the structure of the laminated layer and that EmAP, which is a glycoprotein, has most likely dissociated from this structure during the progressive loss of parasite viability. Thus, EmAP, as it appears in the culture supernatant during the course of in vitro drug treatment, represents a marker which is indicative for the impairment of metacestode viability.

AE is—quantitatively—not regarded as one of the major parasitic diseases. However, the consequences for the individual patient are extremely severe, and the disease leads to death in those patients for whom chemotherapy is unsuccessful in halting parasite growth (18). Therefore, novel compounds should be tested for antimetacestode activity in order to im-

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prove the present treatment protocols. A first step in that direction will be the primary in vitro screening of novel reagents, and the test system based on monitoring EmAP activity appears to be an ideal tool for such studies involving numerous compounds. The activity of this enzyme can be easily determined and quantified using standard ELISA substrate reagents and an ELISA reader (as in this study). Alternatively, EmAP activity could also be qualitatively visualized by dot blot assay, yielding identical results as shown with the ELISA-based approach. In combination with SEM, measurement of EmAP activity in culture supernatants will allow one to obtain fast and reliable results during primary in vitro drug screening using numerous chemotherapeutically interesting compounds without the involvement of costly and time-consuming animal experimentation.

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