Effect of Amphotericin B on Larval Growth of *Echinococcus multilocularis*

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Alveolar echinococcosis is caused by the parasitic cestode *Echinococcus multilocularis*. Benzimidazoles, namely, mebendazole and albendazole, are the only drugs available for the treatment of inoperable alveolar echinococcosis. At present, no therapeutic alternative is available for patients with progressive disease under treatment or for patients who are unable to tolerate the side effects of the benzimidazoles. In addition, benzimidazoles are only parasitostatic for *E. multilocularis*. Thus, new therapeutic options are of paramount importance. In the present study we examined the in vitro effect of amphotericin B on *E. multilocularis* larvae. *E. multilocularis* metacestodes grown in the peritoneal cavities of Mongolian gerbils were transferred into a culture system. Vesicles budded from the tissue blocks and increased in number and size during the first 5 weeks. After 6 weeks drugs were added and deleterious effects on the vesicles were observed macroscopically and microscopically. By use of this in vitro tissue culture model we demonstrated that amphotericin B effectively inhibits the growth of *E. multilocularis* metacestodes. This destructive effect was significantly more rapid with amphotericin B than with the benzimidazoles. Cyclic treatment was effective in suppressing parasite growth. However, amphotericin B appears to be parasitostatic for *E. multilocularis* larvae, and regrowth occurs even after extended periods. In summary, amphotericin B constitutes the first promising alternative for the treatment of alveolar echinococcosis in cases of intolerance or resistance to benzimidazoles. It holds promise as an effective treatment option for otherwise fatal courses of disease.

The larval stage of *Echinococcus multilocularis* causes alveolar echinococcosis (AE), a parasitic disease primarily affecting the liver. Human AE is endemic in regions of Western and Central Europe, Eastern Europe, North America, and Asia. Untreated AE is fatal in over 90% of cases, and surgical resection is often incomplete due to the diffuse infiltration of nonresectable structures.

Benzimidazole carbamate derivatives, namely, mebendazole (MBZ) and albendazole (ABZ), are the only drugs available for the treatment of human AE. A disadvantage of the benzimidazoles is the fact that these drugs are parasitostatic rather than parasitocidal for *E. multilocularis*. Thus, new therapeutic options are of paramount importance. In the present study we examined the in vitro effect of amphotericin B on *E. multilocularis* larvae. *E. multilocularis* metacestodes grown in the peritoneal cavities of Mongolian gerbils were transferred into a culture system. Vesicles budded from the tissue blocks and increased in number and size during the first 5 weeks. After 6 weeks drugs were added and deleterious effects on the vesicles were observed macroscopically and microscopically. By use of this in vitro tissue culture model we demonstrated that amphotericin B effectively inhibits the growth of *E. multilocularis* metacestodes. This destructive effect was significantly more rapid with amphotericin B than with the benzimidazoles. Cyclic treatment was effective in suppressing parasite growth. However, amphotericin B appears to be parasitostatic for *E. multilocularis* larvae, and regrowth occurs even after extended periods. In summary, amphotericin B constitutes the first promising alternative for the treatment of alveolar echinococcosis in cases of intolerance or resistance to benzimidazoles. It holds promise as an effective treatment option for otherwise fatal courses of disease.

**MATERIALS AND METHODS**

*E. multilocularis* metacestodes, originally isolated from humans, were maintained in Mongolian gerbils (*Meriones unguiculatus*) by intraperitoneal injection of minced metacestode tissue. After 6 to 8 weeks the gerbils were euthanized. Metacestodes were isolated from the peritoneal cavity and cut into tissue blocks of 0.5 cm³. After two washings with phosphate-buffered saline, three tissue blocks were placed in each 25-cm³ cell culture flask containing 20 ml of Dulbecco’s modified Eagle medium (Biochrom AG, Berlin, Germany). The culture medium was supplemented with 10% fetal calf serum (PAA Laboratories, Linz, Austria), 200 μg of penicillin/ml, 200 μg of streptomycin/ml, and 10 μg of levofoxacin/ml. Cells of the human liver cell line HepG2 adherent to the bottom of 25-cm³ flasks were grown. Tissue cultures were incubated at 37°C with 5% CO₂. The culture medium was changed thrice weekly, and the cultures were monitored by light microscopy for growth and the integrity of the parasitic vesicles. The basic features of this culture method for the metacestode stage of *E. multilocularis* were first described by Hemphill et al. (16) and Jura et al. (21).

The follow-up parameters recorded were the numbers and sizes of secondary vesicles growing from the tissue block as well as protoscolex formation inside the vesicles. All experiments were performed in duplicate.

**Antihelminthic drugs.** AMB (Biochrom AG) was resuspended in 500 μl of aqua destillata and was used at a concentration of 2.5 μg/ml. ABZ and MBZ (Sigma-Aldrich, Taufkirchen, Germany) as well as ABZ sulfoxide (ABZSO) and ABZ sulfone (ABZSN) (kindly provided by R. J. Horton, SmithKline Beecham, London, United Kingdom) were resuspended in 40 μl of dimethyl sulfoxide (DMSO)/20 ml and were used at a concentration of 1 μg/ml. Control cultures for the benzimidazoles contained 40 μl of DMSO/20 ml.

**Viability testing.** In vivo viability testing was performed by injection of larval tissue into Mongolian gerbils (*Meriones unguiculatus*; age, approximately 3 months). Larval tissue was minced through a sieve with 0.5-mm pores and...
resuspended in Dulbecco’s modified Eagle medium. A total of 0.2 ml of the suspension was injected into the peritoneal cavity of a gerbil by using a 1-ml syringe with a 20-gauge needle. Two gerbils were used for each test. After 6 weeks the gerbils were euthanized, the abdominal cavity was opened, and larval growth was assessed. The proliferation of vesicles in gerbils confirmed that the larval tissue was viable. The testing of viability by injection of tissue into rodents (in vivo viability test) is an established and reliable procedure (20, 31), and the use of animals complied with German federal guidelines (Regulation 706) and the institutional policies of the University of Ulm.

**Statistical analysis.** All experiments were performed in duplicate, and the interassay variation was <10%. The time courses of the number of vesicles in culture are depicted as line charts, and the concentration of AMB in the medium and inside the vesicles is shown in box plots.

**RESULTS**

**Tissue culture.** Metacestode tissue blocks were extracted from gerbils and placed in culture as described above. Budding of vesicles from the tissue blocks started after 3 to 5 days, and the vesicles increased in size during the first 5 weeks before they reached a steady state (Fig. 1). In most vesicles protosco-

FIG. 1. (A) Three metacestode tissue blocks in culture. After 3 to 5 days vesicles start emerging from the tissue blocks (B) and increase in size and number until they reach a steady state after 5 weeks (C).

lices started to develop after 40 to 60 days. The total number of vesicles at steady state varied between 80 and 120. Vesicle diameters slowly increased to up to 10 to 22 mm over 8 weeks. *E. multilocularis* metacestodes could be maintained for >58 weeks in this culture system.

**Destruction of vesicles.** AMB was added at a concentration 2.5 μg/ml to tissue blocks cultured for more than 6 weeks. Disintegration of vesicles was first notable after 1 day, and after 7 days all vesicles were completely destroyed (Fig. 2). The concentration of 2.5 μg/ml was chosen due to an optimal dose-response relationship; lower doses (1.25 and 0.625 μg/ml) caused vesicle destruction to a lesser extent (results not shown). Four benzimidazole derivatives (MBZ, ABZ, ABZSN, and ABZSO) served as controls, and each benzimidazole derivative was used at a concentration of 1 μg/ml. The disintegration of vesicles was first noted after 3 days. After 25 to 29 days all vesicles were completely destroyed. All the different benzimidazole derivatives had similar effects on the metacestode vesicles, but the effect of ABZSN was observed slightly later than those of the other benzimidazoles. When benzimidazoles were used at a concentration of 10 μg/ml, the vesicles were destroyed at a rate similar to that achieved with a concentration of 1 μg/ml (data not shown).

**Cyclic treatment and resurgence of vesicles** AMB was added to metacestode tissue after 10 weeks of culture. Again, destruction of vesicles was observed as soon as 1 day after the start of treatment, and all vesicles were disrupted after 8 days (Fig. 3). AMB was discontinued after 28 days. The resurgence of new vesicles was noted 6 days after discontinuation of the medication, and the vesicles increased in number over 24 days until they reached a steady state at approximately 40 vesicles. At this point, AMB was again added to the metacestode cultures for 28 days. During the second cycle all vesicles were disrupted after 9 days. At the end of the second 28-day cycle, the resurgence of vesicles was protracted until 24 days after the end of treatment. The vesicles were again allowed to grow and reached a steady state at approximately 20 vesicles. A third cycle and a fourth cycle of AMB were given. After the fourth
cycle cultures were observed for another 16 weeks without treatment. No resurgence of vesicles was observed during this time. At the end of the experiment, the metacestode tissue blocks were homogenized and injected intraperitoneally into gerbils. Intraperitoneal growth of metacestodes was observed in all animals.

Regrowth of vesicles from tissue blocks was observed after discontinuation of benzimidazoles (ABZ, MBZ, ABZSO, and ABZSN) as well (results not shown). The benzimidazoles were applied for 28 days prior to discontinuation.

**DISCUSSION**

**The effect of AMB on metacestodes.** The results of the present study show that AMB at the concentration used is highly effective against *E. multilocularis* metacestodes in vitro. The chemotherapeutic effect was assessed by the loss of turgidity and disintegration of metacestode vesicles grown in tissue culture.

The time until metacestode vesicles showed signs of destruction was significantly shorter for AMB than for several benzimidazole carbamates (i.e., MBZ, ABZ, ABZSO, and ABZSN). ABZ is metabolized into ABZSO after resorption from the gastrointestinal tract and is later converted into ABZSN (18, 27). These metabolites were tested because they are believed to be the active components which act on *E. multilocularis* metacestodes in vivo. In our in vitro experiments ABZSO had effects similar to those of ABZ and MBZ, while the destructive effect of ABZSN on metacestode vesicles was slightly delayed (Fig. 2). The effects of ABZSO and ABZSN against *E. multilocularis* metacestodes in vitro were recently investigated (18), and it was shown that both derivatives are absorbed into the vesicles and have similar destructive effects.

We used AMB at a concentration of 2.5 μg/ml because that concentration is analogous to the drug concentrations effective against *Aspergillus* isolates in vitro (12). In an attempt to correlate the in vitro situation with the in vivo situation, Lewis et al. (22) constructed an in vitro infection model capable of simulating the pharmacokinetic parameters for AMB in human serum. Those investigators found in vitro peak concentrations of 2.4 μg/ml, corresponding to a dose of 1 mg/kg of body weight every 24 h. However, due to the accumulation of AMB in different organs, the optimal dosing for in vivo use cannot be determined in an in vitro setting alone, and lower concentrations in serum may be sufficient in vivo. Furthermore, the defensive role of the host immune system adds to the containment of parasite growth, an element not simulated by our in vitro setting. Thus, animal experiments are needed to determine the optimal dosing for in vivo use.

**Long-term effect of AMB.** The ability to cultivate metacestodes over a prolonged time of up to 58 weeks enabled us for the first time to study time kinetics. The culture system used in the present culture system appears suitable for the evaluation of viability. Our experiments yielded three major observations. First, the number of treatment cycles influenced the time until vesicles reemerged from the metacestode tissue block (Fig. 3).

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**FIG. 2.** Treatment of metacestode vesicles with AMB and benzimidazoles (ABZ, MBZ, ABZSO, and ABZSN). Metacestodes were cultured in 25-cm² flasks together with the human liver cell line HepG2. Medium was exchanged thrice weekly. Cultures were monitored by light microscopy for growth and the integrity of the parasitic vesicles. All experiments were performed in duplicate, and the average number of vesicles is depicted (interassay variation, <10%). Note that the vesicles treated with AMB start disintegrating after 1 day, while disintegration was first noted after 3 days in the vesicles treated with the benzimidazoles. The slope of the destruction curve is steeper for AMB than for the benzimidazoles. Total destruction of vesicles is observed after 7 days of AMB treatment and after 25 to 29 days of benzimidazole treatment.
Second, after each additional treatment cycle the number of vesicles reached a lower steady state. The steady state in vesicle formation could be assumed after short periods of time (2 weeks), because previous experiments (data not shown) with a longer drug-free period had revealed that this steady state was maintained for more than 6 weeks.

Third, repeated application of AMB resulted in the loss of vesicle formation from the tissue blocks. However, even after 16 weeks without apparent activity (i.e., without the formation of vesicles), the parasitic structures inside the tissue block remained viable and were able to proliferate when they were injected into gerbils. In vivo viability testing by injection of tissue into rodents is an established and reliable procedure (4, 14, 20, 31, 35). Thus, these results show that despite the high degree of efficacy of AMB against *E. multilocularis* metacestodes, this drug acts only parasitostatically even after prolonged and repeated application. The lack of parasitocidal activity may be the consequence of a failure of AMB to diffuse into the inner part of the tissue block. This interpretation is stressed by the fact that the longer that AMB is applied, the longer the time that is needed for regrowth of vesicles from the tissue block (Fig. 3), and regrowth presumably occurs from the few surviving inner cells. This situation may also reflect the in vivo situation, in which drugs are unable to kill the parasite due to insufficient diffusion (of drugs) into inner parts of the lesion.

The design of a culture model appears to be essential for the testing of the drug effect. In the tissue culture system used in this study, we tested the effect of drugs on the vesicles together with the metacestode tissue blocks. Hereby, we were able to maintain metacestodes in culture over extended periods. Assessment of the long-term effect is important in order to differentiate parasitostatic from parasitocidal drug activity. Other investigators have previously tested the effects of benzimidazoles on vesicles after separation from the tissue blocks (18, 20). On the basis of those models, the investigators came to the conclusion that benzimidazoles have a parasitocidal effect in vitro. On the other hand, however, benzimidazoles lack this parasitostatic effect in vivo (5, 6, 36). This apparent discrepancy between the in vitro activity and the in vivo activity may be explained by the type of in vitro culture systems, in which isolated vesicles are used. In the presence of metacestode tissue blocks, we observed regrowth of vesicles after discontinuation of benzimidazoles. Therefore, in our model, as was observed in vivo, benzimidazoles exhibit only a parasitostatic effect. This demonstrates that metacestode tissue blocks are essential for evaluation of the long-term effects of drugs on the parasite. The parasite may remain viable in the tissue blocks long after the vesicles have been destroyed.

**Mechanism of action of AMB.** AMB has been the most important fungicidal agent for decades. This drug is naturally derived from *Streptomyces nodosus* (10). Although its mode of action is not yet fully understood, the most important mechanism appears to be the selective and irreversible binding to sterols (ergosterol and cholesterol) in cell membranes, thereby forming transmembrane channels (2, 8, 15). Furthermore, AMB was shown to form stable complexes with membrane phospholipids (9, 25, 34), to perturbate the fluidity of membranes (1, 17), and to have an effect on anion transport and membrane-bound enzymes (3, 7, 33).

It was recognized later that AMB also has antiparasitic ac-
tivities against Leishmania and Trypanosoma (15, 30, 37). The spectrum of activity is now extended by its effect on E. multilocularis metacestodes, as shown in the present study. The elucidation of its mechanism of action on this parasite will be a task for future studies. We may speculate that, in analogy to the action of AMB on fungi, the formation of complexes with lipids could be the destructive mechanism on E. multilocularis larvae as well. Membranes of E. multilocularis metacestodes were shown to contain major amounts of phospholipids and neutral lipids (cholesterol, triacylglycerides, and steryl esters) (28, 29). Thus, various metacestode components could potentially interact with AMB.

Although AMB proved to be highly efficient against E. multilocularis metacestodes in vitro, this drug is known for its serious side effects, such as severe nephrotoxicity and hypokalemia. Thus, the minimal effective dose will have to be determined in vivo. Another treatment option for the reduction of nephrotoxicity would be cyclic dosing. Our experiments show that cyclic dosing with intervals of several weeks of a drug holiday may be feasible and may result in sustained suppression of vesicle growth. Vesicles did not reemerge during periods of drug holidays. Although it was not examined in this study, one could speculate that the combination of AMB with other drugs (e.g., the benzimidazoles) might lead to an AMB dose reduction. It should be emphasized that AMB does not have the potential to replace the benzimidazoles as standard treatment for AE, because in the majority of patients parasite growth can be successfully controlled with life-long benzimidazole treatment. AMB might be considered an alternative drug only for patients who cannot tolerate benzimidazoles and who will otherwise progress to fatal courses of disease.

Culture system. Various models for cultivation of metacestode tissue have been described by others (16, 21). The cultivation of metacestodes constitutes a suitable test system for assessing the effects of antiparasite drugs, because the metacestodes cultivated in vitro all contain the important metacestode structures and thus show strong similarities to the situation in vivo. It was shown previously that the surface of the metacestode vesicle is composed of an acellular laminated layer, which covers the entire larva (13). This laminated layer protects the parasite from host defense mechanisms. In vitro, this laminated layer is formed after 13 days (13). The laminated layer surrounds the multicellular laminar layer and the developing protoscolices. The present culture system may be taken as a model for the situation in vivo, because the growth of vesicles from the tissue block in vitro resembles the centripetal expansion of metacestode tissue in vivo. Furthermore, the steady state of vesicle growth in vitro over prolonged periods of time resembles the chronic persistence of the parasite in the liver. In the present experiments vesicles were grown for at least 6 weeks before medication was added.

Conclusions. Our in vitro system for cultivation of metacestodes constitutes a suitable model for testing of antiparasitic drugs. AMB effectively inhibits the growth of E. multilocularis larvae in vitro and holds great promise for the treatment of otherwise untreated AE. Its effect was more rapid than those of the benzimidazoles. However, under the present conditions AMB exerts only a parasitostatic effect on E. multilocularis metacestodes in vitro. Future studies must show whether treatments with combinations of existing or new drugs are more efficient and possibly parasitocidal. The optimal dosing for maximal efficacy and minimal side effects will have to be determined in vivo studies.

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