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Hepatic Tissue Culture Model for Study of Host-Parasite Interactions in Alveolar Echinococcosis

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An in vitro model for growth and differentiation of the metacestode tissue of the tapeworm *Echinococcus multilocularis* is described. This model simulates the organotropism of the parasite toward the liver of the intermediate host. In the presence of collagen-embedded primary hepatocytes from rats and humans, which can be kept in culture for 2 to 3 months, the parasitic vesicles grew by exogenous budding and multiplied about 12-fold within 3 weeks. In contrast, without the hepatocytes, the metacestodes rapidly degenerated. Development of protoscolices was seen only in the presence of rat hepatocytes but not in coculture of the metacestodes with hepatocytes of human origin, thus reflecting the in vivo situation during infection of rodents and in alveolar echinococcosis in humans. The experiments indicated that growth of the metacestodes and development of protoscolices depended on soluble low-molecular-weight factors released by the hepatocytes. The in vitro-grown metacestodes did not differ morphologically from the larvae found in infected intermediate hosts, and their infectivity was completely maintained. This report describes the first in vitro model of alveolar echinococcosis and will be the basis for future studies on host-parasite interactions of this important zoonosis.

Alveolar echinococcosis caused by the larval stage of the tapeworm *Echinococcus multilocularis* is one of the most dangerous zoonoses prevalent in the northern hemisphere (16). Without treatment, this parasitosis is lethal in 94% of patients within 10 years of diagnosis (19). Surgery and chemotherapy have considerably improved the prognosis, but because of the infiltrative, tumor-like growth of the parasite, complete resection of the metacestode tissue is frequently impossible. Furthermore, chemotherapeutic drugs are not parasitocidal but parasitostatic only (4).

Infections occur by ingestion of eggs, which are released into the environment with the feces of foxes or dogs carrying the adult worms in their small intestine. After oral ingestion by the natural intermediate hosts, i.e., small rodents, or accidentally by humans, the eggs hatch in the stomach or small intestine and oncospheres are released, which subsequently penetrate the small intestine and reach the liver via the portal blood flow. Here, the metacestodes of the *E. multilocularis* tissue develop by asexual metagenesis (22). The infiltratively growing metacestode tissue consists of numerous small vesicles, which are

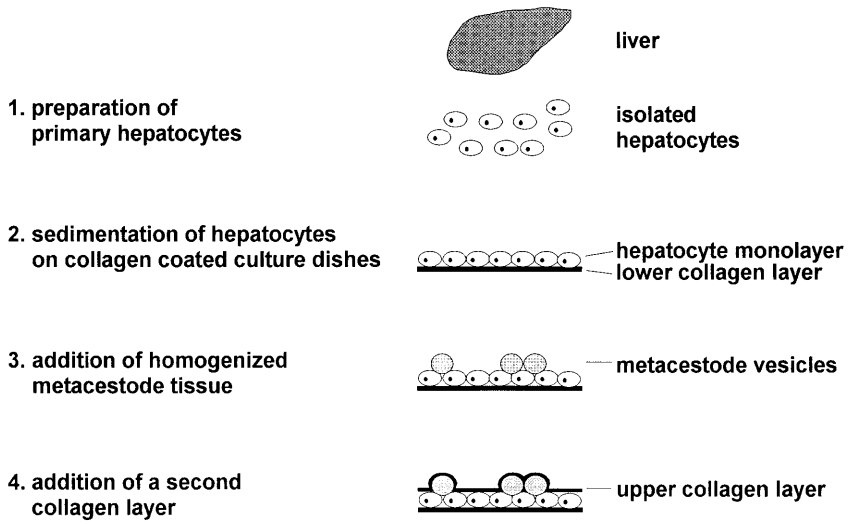

FIG. 1. Schematic diagram of the culture system for propagation of echinococcal metacestode tissue on primary hepatocytes.

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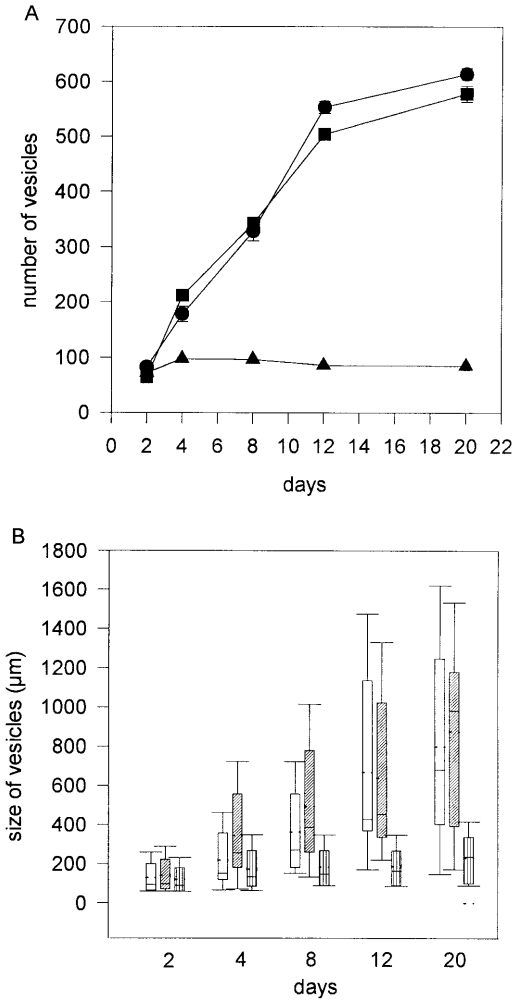

FIG. 2. (A) Multiplication of *E. multilocularis* vesicles over a period of 3 weeks in the presence of rat hepatocytes (circles) and human hepatocytes (squares). No growth was seen in the absence of hepatocytes (triangles). The mean and standard deviation for three cultures infected with the same parasite suspension are given. The experiments were repeated at least 10 times, and a six- to sevenfold increase in the number of vesicles was observed in every experiment. (B) Increase in the diameter of the vesicles. The data are presented as a Whisker plot. The boxes indicate the 25th and 75th percentiles, the solid and dotted lines within the box represent the median and mean values, respectively; and the 10th and 90th percentiles are shown by capped bars. The open boxes represent the growth on rat hepatocytes, the hatched boxes show the growth in the presence of human hepatocytes, the striped boxes indicate the growth kinetics in the absence of hepatocytes. Presentation and statistical evaluation of the data were performed with SigmaPlot software (Jandell Scientific, Erkrath, Germany).

embedded in a dense stroma of connective tissue. In central areas of the parasitic tumor, necrotic masses and calcifications are observed (5). In rodents, protoscolices develop within the vesicles by asexual multiplication of the metacestode as early as 2 to 4 months after infection. In completely developed protoscolices, a corona of hooks is observed in the invaginated rostellum (21). Interestingly, formation of protoscolices is not observed in infected humans, suggesting that the host factors contributing to the development of protoscolices in rodents are not present or are not appropriate in humans.

Studies of the host-parasite relationship in alveolar echinococcosis are limited by the lack of suitable in vitro models. Reports about established echinococcal cell lines are still controversial (7). Furthermore, the metacestode tissue is unable to grow and to differentiate into protoscolices in vitro. Previous studies suggested that growth factors present in human ascites fluid and embryonic extracts of intermediate hosts or provided by a colon carcinoma cell line are required for in vitro growth of *E. multilocularis* metacestodes (10, 15). In the present study, we investigated whether the limitations of parasitic growth and differentiation in vitro could be overcome by using a culture system which simulated the situation during infection in vivo. We established a tissue culture system for the parasitic tissue with primary rat and human hepatocytes. Under these conditions, growth of the metacestode tissue and development of protoscolices could be achieved.

MATERIALS AND METHODS

Isolation and in vitro cultivation of metacestodes of *E. multilocularis*. Metacestode tissue of *E. multilocularis* was continuously kept in Mongolian gerbils (*Meriones unguiculatus*) by intraperitoneal infection with homogenized tissue. The parasite strain was isolated as infective eggs from the intestine of a fox from the Swabian Mountains, Germany, and further propagated in *M. unguiculatus*. After 6 to 8 weeks, the tumor mass was isolated, homogenized in phosphate-buffered saline (PBS), and passed through a plastic sieve and then through a nylon mesh (pore size, 100 μm). The larval tissue was washed three times with 50 ml of PBS (pH 7.5) and sedimented for 20 min. The vesicles were shown by light microscopy to contain no protoscolices. The sediment was resuspended in 3 ml of PBS, and 30 to 100 μl of this suspension containing 50 to 150 vesicles was added to the culture dishes of the hepatocyte cultures.

Isolation of primary rat and human hepatocytes. For isolation of rat hepatocytes, a modification of the two-step method described by Seglen (20) was applied, using female Lewis rats weighing 200 to 250 g. The rats were anesthetized with ether. Following midline incision and cannulation of the portal vein, the liver was perfused in situ with 400 ml of Ca^{2+}-free Krebs Ringer buffer (KRB) for 10 min and then with 200 ml of KRB containing 21.5 U of collagenase type II (Biochrom, Berlin, Germany) plus 9 ml of 11-mol/liter $CaCl_2$ for 8 min. All perfusates were equilibrated with 95% oxygen–5% carbon dioxide at 37°C. Following perfusion, the liver capsule was gently removed and the dissolved liver tissue was washed three times with ice-cold KRB after being filtered through a nylon mesh (pore size, 100 μm). The parenchymal cell suspension was purified by Percoll gradient centrifugation (Pharmacia, Heidelberg, Germany). The Percoll gradient was prepared by adding 1.2 ml of Hanks balanced salt solution to 10.8 ml of Percoll. This procedure was followed by the addition of 12.5 ml of washing buffer containing 25% of the cell pellet obtained from the previous steps. After gentle mixing of the resulting cell suspension, the cells were centrifuged again at $50 \times g$ for 5 min at 4°C. The remaining cell pellet was used for the following experiments. The viability of the cells ranged between 85 and 92% as assessed by trypan blue exclusion. On average, 3×10^8 to 5×10^8 cells were obtained from one isolation procedure.

Human hepatocytes were isolated from specimens obtained from patients undergoing hepatic resections for hepatic cancer. Pieces were taken immediately after the resection from the safety margin of 4 to 5 cm resected together with the nodular tumor. The specimens were transferred to ice-cold KRB without Ca^{2+}. Vessels visible on the cut surface were cannulated. Perfusion was started with 400 ml of Ca^{2+}-free KRB for 8 min followed by 200 ml of KRB containing 21.5 U of collagenase type IV (Biochrom) plus 9 ml of 11-mol/liter $CaCl_2$ for 12 min. The collagenase perfusate was recirculated, and perfusion was performed at a rate of 50 ml/min. All perfusates were equilibrated with 95% oxygen–5% carbon dioxide and warmed to 37°C. The resulting cell suspension was filtered through a nylon mesh (pore size, 100 μm) and washed three times with cold KRB. Hepatocyte viability ranged from 90 to 95%. On average, 6×10^7 cells were obtained from one isolation procedure.

Hepatocyte cultures. Primary human and rat hepatocytes were enclosed within two layers of collagen as described previously (2, 8) with some modifications (Fig. 1). At 30 min after surface coating of 60-mm petri dishes with collagen, hepatocytes were seeded at a density of 2×10^6 cells per dish. At 4 h following seeding and attachment of the cells, the culture medium (Williams' E; Biochrom) was removed along with nonadherent cells. The medium was again changed after 2 h. The next day, the medium was aspirated and 30 to 100 μl of homogenized metacestodes was added before a second layer of liquid and ice-cold collagen was applied. For control experiments, collagen was added without first being inoculated with parasites. After gelation of this second matrix layer, a sandwich configuration with two layers of hydrated collagen gel was formed, reflecting in vitro the in vivo bipolar hepatocellular enclosure within the matrix of the space of Disse. At 30 min following gelation of the second layer of matrix, culture medium was placed on top. The pH of the collagen was adjusted to 7.4 with a 10× Dulbecco's modified Eagle's medium (Biochrom) concentrate, which was diluted with the collagen solution at a ratio of 1:10. The total volume of cell culture supernatant was 4 ml, including the hydrated collagen matrix volume of 2 ml.

Culture media were supplemented with 10% (vol/vol) fetal bovine serum

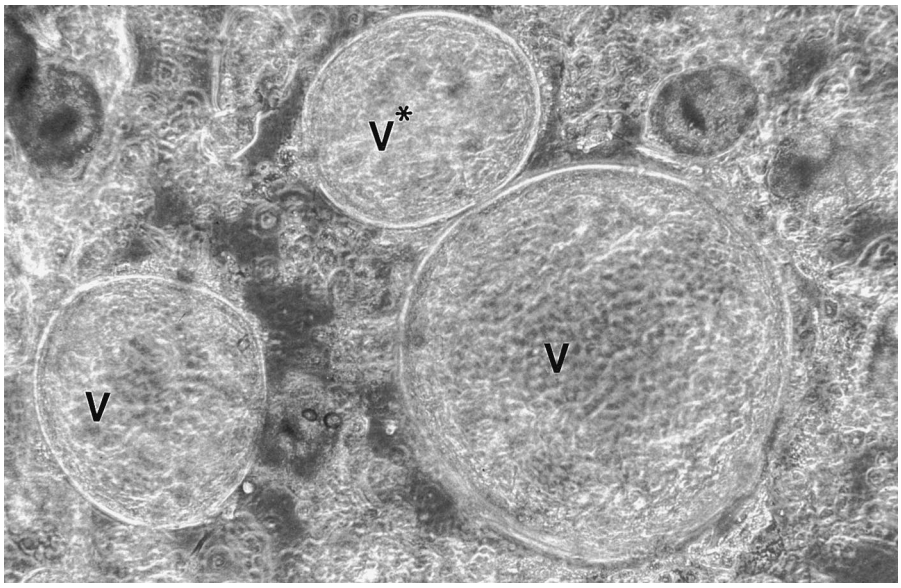

FIG. 3. *E. multilocularis* metacestodes (V) grown in the presence of a monolayer of rat hepatocytes. V* indicates a vesicle generated in vitro by exogeneous budding. Magnification, ×35.

(Williams' E), 9.6 μg of prednisolone 9.6 per ml, 0.014 μg of glucagon (Novo, Mainz, Germany) per ml, and 0.16 U of insulin (Hoechst, Frankfurt, Germany) per ml. Penicillin (200 U/ml) and streptomycin (200 μg/ml; Biochrom) were also added. The culture medium was replaced with fresh medium every 24 h. The removed culture supernatants were collected and stored at −20°C until further analysis of the rat albumin content. Rat tail collagen was prepared by the method described by Elsdale and Bard (6). A final concentration of 1.11 mg of collagen per ml was used for coating the petri dishes (diameter, 60 mm; Greiner, Frickenhausen, Germany).

Alternatively, metacestodes were applied within dialysis bags to the hepatocyte culture. Dialysis membranes (Spectra/Por) with molecular weight cutoffs of 3,500 and 6,000 to 8,000 were purchased from Roth, Karlsruhe, Germany. The membranes were soaked in sterile water for 3 h prior to use and filled with 1 ml of homogenized metacestodes in supplemented Williams' E medium.

Albumin assay. To determine the viability and functional integrity of the hepatocyte cultures, collected media were analyzed for rat serum albumin by an enzyme-linked immunosorbent assay, which was described in detail elsewhere (3). Chromatographically purified rat albumin (Sigma, Deisenhofen, Germany) was used as standard. Peroxidase-conjugated antibody to rat albumin was purchased from Cappel, Cochranville, Pa. The A_{492} was measured in a Titertek Multiscan (no. MCC 1340; Flow Laboratories, Meckenheim, Germany). The albumin production profile during the parasite cultivation period of 4 to 6 weeks corresponded to that described previously (3).

Transmission electron microscopy. For electron microscopy, cell cultures were fixed in 2% glutaraldehyde in cacodylate buffer (pH 6) for 12 h at 4°C. The cultures were then rinsed in 0.1 mol of sodium cacodylate buffer per liter, postfixed in 1% osmic acid for 1 h, dehydrated in graded ethanol, and finally embedded in agar resin. Ultrathin sections were stained with an aqueous solution of uranyl acetate-lead citrate and examined with an electron microscope (Zeiss 10B).

Light microscopy. Metacestodes were fixed in 4% formalin for 24 h and embedded in paraffin. Sections (5 μm) were stained with hematoxylin-eosin.

RESULTS

Growth of *E. multilocularis* metacestodes on primary rat hepatocytes. To evaluate whether primary rat hepatocytes can be used as an in vitro model for growth and differentiation of *E. multilocularis* metacestodes, parasitic tumours isolated from intraperitoneally infected gerbils and homogenized to a particle size of ≈100 μm were applied to freshly established hepatocyte cultures. Primary hepatocytes and parasitic tissues were both embedded in a collagen bilayer, which is essential to keep the hepatocytes live and functional over a period of several weeks and months (1). This approach was also advantageous for the cultivation of the parasitic vesicles, which were not adherent to the hepatocytes. The embedding in collagen thus improved the handling of the cultures, since the vesicles were not removed by the daily change of the cell culture medium.

The number and the size of parasitic vesicles cultured under these conditions were determined daily. As shown in Fig. 2A, in a typical experiment the number of vesicles greatly increased from 52 at the time of inoculation to more than 640 after 3 weeks. The increase in the number of vesicles was due to exogeneous budding of small daughter vesicles from larger parental vesicles (Fig. 3). In contrast, only a moderate multiplication of the metacestode tissue during the first few days was observed when the hepatocytes were omitted but all other culture conditions remained identical. After 6 days under these culture conditions, the cysts began to degenerate, and after 18 days, no intact vesicles were present. In the presence of hepatocytes, the average size of the parasitic vesicles increased about sevenfold from a mean diameter of 127 μm at the time of inoculation to a mean diameter size of 960 μm after 3 weeks. The vesicles maintained without hepatocytes in the control experiments showed no significant increase in size during the same time (Fig. 2B). It should be emphasized that the individual sizes of different vesicles varied considerably in older cultures and that vesicles with sizes between 120 and 1660 μm were found after 3 weeks, thus reflecting the presence of old, large vesicles, which were from the inoculated parasitic tissue, and small, younger vesicles asexually generated during the incubation period by exogeneous budding (Fig. 3).

The hepatocyte-parasite cultures could be kept for at least 2 months with continuing growth and budding of the parasitic vesicles. However, after 3 to 4 weeks, the upper collagen layer became perforated by the largest vesicles and a considerable number of vesicles was released to the culture medium. Under these conditions, further reliable evaluation of the growth kinetics as shown in Fig. 2 and 4 was not possible, since vesicles in the supernatant were removed by the daily medium changes.

Development of protoscolices. In rodents, protoscolices develop within echinococcal vesicles by asexual metagenesis. The preparation and homogenization procedure of metacestode tissue grown in gerbils released a considerable number of pro-

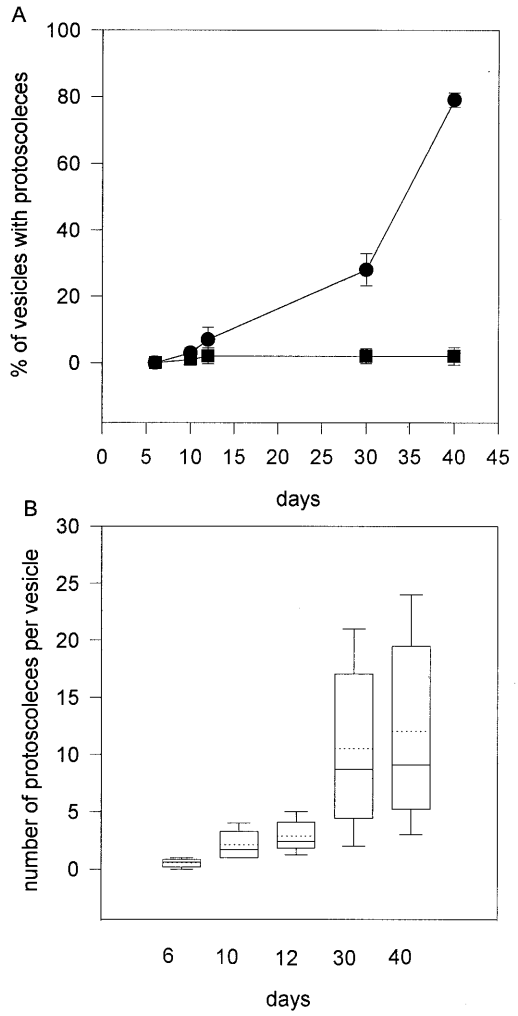

FIG. 4. (A) Development of protoscolices within *E. multilocularis* vesicles over a period of 40 days in the presence (circles) or absence (squares) of rat hepatocytes. The mean and standard deviation for three cultures infected with the same parasite suspension are given. (B) Number of protoscolices per cyst. The data are presented as a Whisker plot. The boxes indicate the 25th and 75th percentiles; the solid and dotted lines within the box represent the median and mean values, respectively; and the 10th and 90th percentiles are shown by capped bars. Presentation and statistical evaluation of the data were performed with SigmaPlot software.

toscolices from the vesicles and could subsequently not completely be removed from the metacestodes used for inoculation of the primary hepatocyte cultures. However, no cysts with internal protoscolices were observed in the inoculum, as carefully monitored by microscopy. Interestingly, the rostellum of the released protoscolices evaginated within the first 3 days after inoculation and became highly motile as observed by microscopy. The evaginated protoscolices began to degenerate after 8 days, and the vast majority were absent after 20 days. However, less than 5% of the protoscolices maintained their motility during the cultivation period of 40 days.

After 2 weeks, inclusions became visible in a few vesicles, suggesting that protoscolices had been developed. After 40 days of cocultivation with rat hepatocytes, 80% of the cysts exhibited these inclusions (Fig. 4A), and mean of nine inclusions per vesicle were observed (Fig. 4B). No inclusions were found in vesicles cultivated without hepatocytes. Hematoxylin-eosin-stained thin sections of isolated vesicles confirmed the presence within the vesicles of invaginated protoscolices, which demonstrated a fully developed corona of hooks in the invaginated rostellum (Fig. 5A).

Parasitic growth in the presence of primary human hepatocytes. In a subsequent experiment, we examined the capacity of primary human liver cells to stimulate the growth and differentiation of echinococcal metacestodes. Similar to the results obtained for rat hepatocytes, the echinococcal vesicles multiplied about 13-fold within 3 weeks (Fig. 2A) and the mean diameter size increased about 6-fold when the parasite was grown on primary human hepatocytes (Fig. 2B). However, during the incubation period of 5 weeks, no development of protoscolices was observed (Fig. 5B). These results are in accordance with the growth characteristics of the parasite in human echinococcosis, where the proliferation of the metacestodes occurs without the development of protoscolices.

Analysis of the infectivity and morphology of the in vitro-grown parasites. To analyze the infectivity of the metacestodes, we isolated single in vitro-grown vesicles from 3- and 6-week-old rat and human hepatocyte culture and injected them intraperitoneally into gerbils. The gerbils were sacrificed after 11 weeks, and a tumor mass was detected in the peritoneum. Microscopic analysis showed a regular laminated and germinal layer, and numerous protoscolices were present within the metacestode tissue. Thus, macroscopic and microscopic examination demonstrated results comparable to those found in parasitic tumor masses during continuous passage of *E. multilocularis* metacestodes in gerbils (data not shown).

To further confirm the integrity and regular morphology of the in vitro-grown metacestodes, parasitic vesicles were subjected to transmission electron microscopy. As shown in Fig. 6, the metacestodes newly developed in the in vitro culture system exhibited the characteristic laminated layer. This extracellular fibrous substance was infiltrated by the microvilli, which are derived from the outer surface of the tegument. Dark-stained undifferentiated cells and syncytial cells, together with glycogen-storing cells, formed the cyst wall. In other areas, muscle cells were also present in the cyst wall (18 and result not shown). Remarkably, there was no direct contact between the laminated layer of the parasite and the surrounding hepatocytes, suggesting that this substance does not mediate the adhesion of the parasitic tissue to the host cells.

Characterization of host factors controlling parasitic growth. To analyze whether growth and differentiation require direct association of the parasite with hepatocytes or whether these processes are controlled and directed by soluble factors secreted into the culture medium by hepatocytes, we separated the echinococcal vesicles from the rat hepatocytes with an additional collagen layer. In an alternative approach, metacestodes and hepatocytes were separated by inclusion of the parasites in dialysis bags (molecular weight cutoff, 12,000 to 14,000), which were layered on the hepatocyte cultures. As in the experiments described above, the metacestodes proliferated 7-fold during an incubation period of 3 weeks and the average diameter of the vesicles increased by a factor of 11 in both experimental approaches. Furthermore, we again observed the generation of protoscolices (data not shown). These experiments suggested that soluble factors secreted by the hepatocytes provided the basis for parasitic growth and development of protoscolices. In a subsequent experiment, we used dialysis membranes with different void volumes for cultivation of metacestodes on hepatocyte cultures. A void volume of 6,000 to 8,000 was still sufficient to allow parasitic growth and protoscolese development, whereas no growth of parasitic tissue was observed in dialysis bags with a void volume of 3,500,

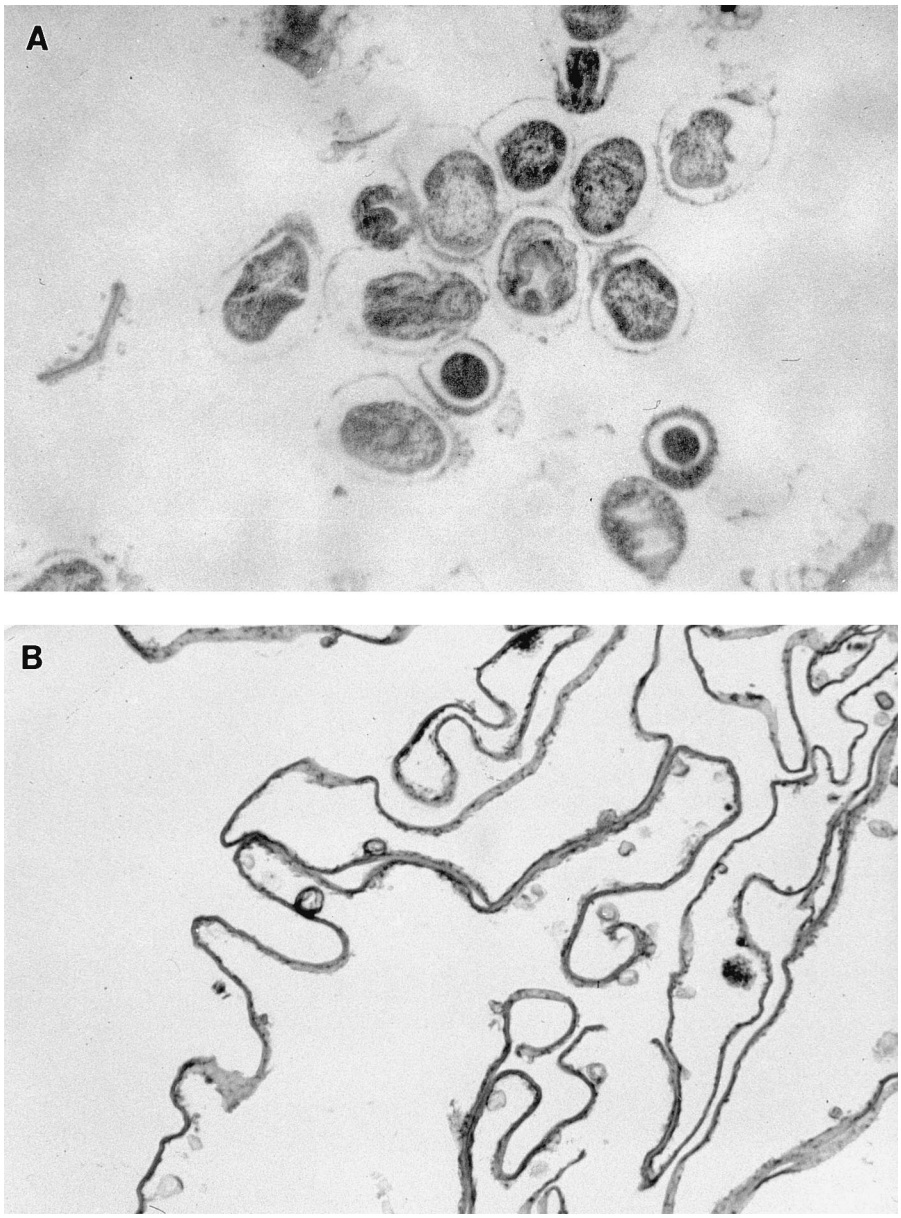

FIG. 5. Light microscopy of thin sections from vesicles of a 40-day-old culture. Development of protoscolices in cysts was observed only in the presence of rat hepatocytes (A) and not in the presence of human hepatocytes (B). The hyaline membranes of the cyst wall are shown in panel B. Magnification, ×100 (A) and ×35 (B).

suggesting a low molecular weight of the growth factor(s) which stimulates parasitic growth and differentiation. However, transfer of supernatants from hepatocyte cultures to metacestodes kept in the absence of hepatocytes failed to stimulate parasitic growth, suggesting a very short half-life of the factor(s).

DISCUSSION

To understand the basis of host-parasite interactions at the molecular level, in vitro models are indispensable. Such models have been established for several parasitic diseases and have introduced important insights into the biology of the parasites and mechanisms of host-parasite interaction at the molecular level (for examples, see references 12, 13, and 17). Although there have been recent advances in some aspects of the biochemistry and molecular biology of *E. multilocularis*, the basis of the host-parasite interaction in alveolar echinococcosis is far from being understood, and the role and significance of parasite antigens, investigated over the past few years by biochemical and recombinant DNA techniques (for a review, see reference 14), in the biology of *E. multilocularis* and in the pathogenesis of echinococcal disease await further clarification. Such studies of alveolar echinoccosis are hampered mainly by two factors: (i) *E. multilocularis* has a complex life cycle involving both carnivores and small rodents, and the isolation and handling of the adult worms living in the intestine of the final hosts are difficult and dangerous and therefore are only limited to a few laboratories with the appropriate facilities; and (ii) no in vitro model that allowed studies on the

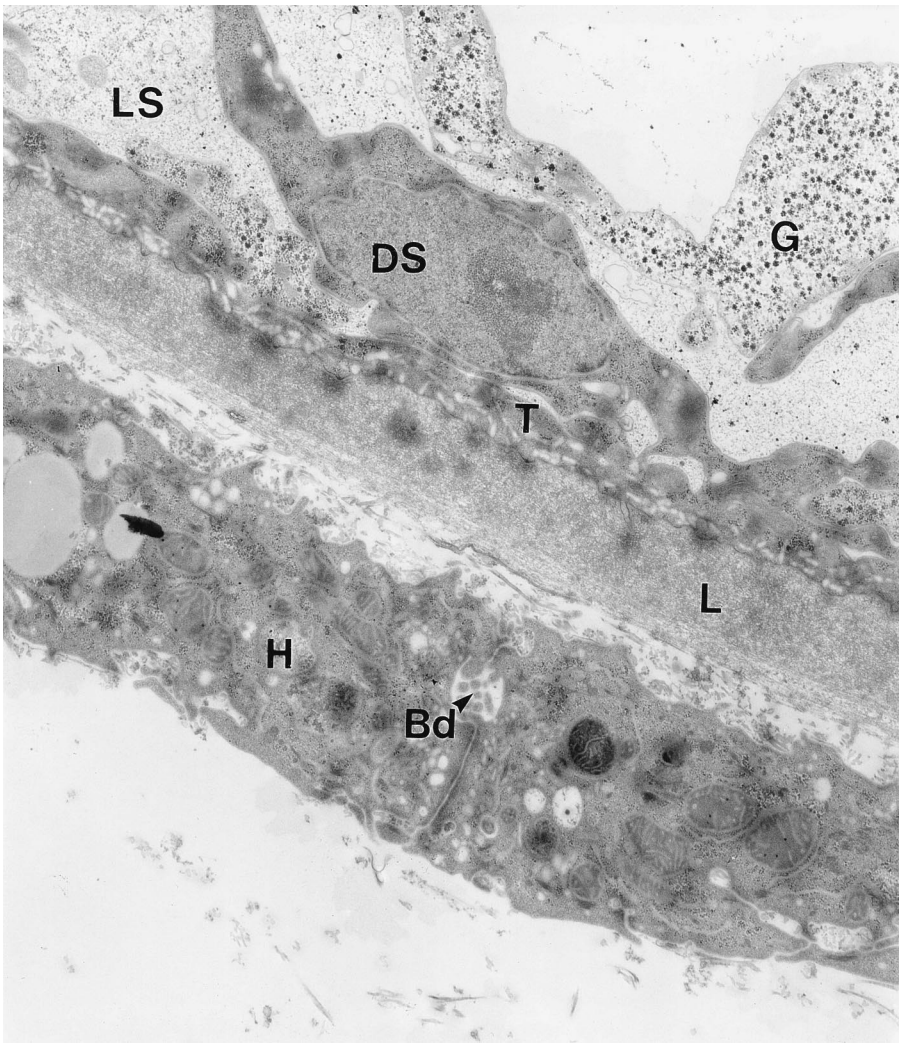

FIG. 6. Electron microscopy of an ultrathin section of rat hepatocytes and a vesicle generated by exogenous budding in vitro. H, hepatocyte; L, laminar layer of the *E. multilocularis* cyst; T, tegument; DS, dark-stained undifferentiated cell; LS light-stained undifferentiated cell; G, glycogen-storing cell. For details of echinococcal histology, see reference 18. Note the formation of a bile duct (Bd) by two hepatocytes. Magnification, ×6,000.

interaction of the parasite with the primary affected organ, i.e., the liver of the intermediate host, has been available until now. Several attempts have been undertaken to establish in vitro culture conditions for the growth of the metacestode tissue. In 1957, Rausch and Jentoft described the cultivation of *E. multilocularis* vesicles in the presence of human ascites fluid and embryonic extracts of intermediate hosts (15). Recently, Hemphill and Gottstein (10) reported that growth of *E. multilocularis* metacestodes and development of protoscolices depended on the presence of a colon carcinoma cell line. These data suggested that factors present in the embryonic extract or provided by certain carcinoma cell lines are required for growth and differentiation of the parasite.

For the first time, we were able to stimulate the in vivo situation of alveolar echinococcosis by an in vitro tissue culture system of primary hepatocytes from intermediate hosts. Our results indicated that the development of the parasite is identical to that observed in experimentally infected animals. The differences seen in the development of protoscolices when rat or human hepatocytes were used as the feeder cell layer support the idea that the results obtained from the in vitro system can be directly applicable to infection in vivo. Protoscolices develop only in small rodents and are not produced in humans suffering from alveolar echinococcosis. The same situation was observed in the in vitro system described here, since formation of protoscolices was absent when human hepatocytes were used. These data could indicate that human hepatocytes lack the production of certain growth factors or, alternatively, that the structures of the human growth factors are not appropriate for induction of the development of protoscolices. Furthermore, it should be emphasized that the same growth characteristics described in this communication were observed for three other *E. multilocularis* isolates, indicating the general usefulness of the tissue culture model (data not shown).

Interestingly, we observed in the tissue culture model that invaginated protoscolices released from the echinococcal cysts during preparation of the tumor mass and already present in the inoculum evaginated and became highly motile in the first days of coculture with rat and human hepatocytes. This phenomenon seemed unusual, since evagination and high motility are not observed in the protoscolices living within the cysts of the intermediate host but, rather, are characteristics of proto-

scolices which differentiate into the adult worm after ingestion by the final host. It is thought that evagination of the protoscolices is stimulated by the bile salts present in the intestine (11). Therefore, we assume that the hepatocytes may produce the bile and thus stimulate the evagination of the protoscolices after release from the cysts. The observation that the collagen-embedded hepatocytes form bile ducts, as shown in Fig. 6, is in accordance with this view.

The tissue culture model of *E. multilocularis* metacestodes with primary hepatocytes from intermediate hosts is the basis for future investigations of host-parasite interactions in alveolar echinococcosis. In the present study, we could demonstrate that a soluble growth factor(s) secreted by primary hepatocytes of the intermediate hosts directs the development of the parasitic tissue. The identification of the growth factor(s) responsible for the development of the parasite and the identification of the corresponding receptors in the parasitic tissue could provide the basis for new therapeutic concepts. Furthermore, we are trying to expand the culture model and include endothelial cells and tissue macrophages, to more closely resemble the in vitro situation. This should enable us to analyze more closely any alterations in cytokine expression to understand the extraordinary ability of echinococcal larvae to control the immune defense mechanisms of the intermediate host (9). Finally, genetic manipulation and transformation of single echinococcal cells should become easier to perform, since the tissue culture model will enable the propagation of the mutagenized cells and the resulting tissue.

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