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Complete Development of *Cryptosporidium parvum* in Bovine Fallopian Tube Epithelial Cells†

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Cryptosporidium parvum **is a coccidian parasite responsible for causing protracted and life-threatening diarrheal illness in immunosuppressed humans, especially patients with AIDS. The lack of medications effective in treating people suffering from cryptosporidiosis has prompted the development of in vivo and in vitro models for this disease. This study is the first to demonstrate that *C. parvum* can complete its entire life cycle (from sporozoite to infective oocyst) in a primary culture of bovine fallopian tube epithelial (BFTE) cells. Scanning and transmission electron photomicrographs were used to detail the ultrastructure of individual parasitic stages. Successful infections were produced by inoculating cell cultures with either oocysts or purified sporozoites. Infection of BFTE cells with *C. parvum* closely paralleled in vivo infections with regard to host cell location and chronology of parasite development. Infecting BFTE cells with sporulated oocysts provided a reproducible and quantitative cultivation system with significantly ($P \leq 0.001$) higher infection rates than in Madin-Darby canine kidney cells. Oocysts produced in BFTE cells were infective for immunosuppressed adult C57BL/6N mice. Cultivation of *C. parvum* in BFTE cells will facilitate the study of interactions between parasites and host cells as well as provide a reliable system for evaluating anticryptosporidial compound efficacy.**

During the past two decades, *Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae) has gained recognition as a prominent enteropathogen of humans. This protozoan parasite was first isolated from the intestinal tracts of human patients in 1976 (18, 19). The disease is unrelenting and life threatening in immunocompromised people, especially those with AIDS (5, 24). In late March and early April 1993, an epidemic of cryptosporidiosis occurred in Milwaukee, Wis., which caused clinical disease in an estimated 403,000 people, thus confirming the importance of this parasite as a waterborne pathogen (17). It was the largest documented outbreak of any waterborne disease to occur in the United States.

Despite the widespread evaluation of hundreds of different modalities in various laboratories throughout the world, there are still no prophylactic or therapeutic compounds with reliable anticryptosporidial activity. In vivo and in vitro models would facilitate the identification of efficacious drugs against *C. parvum*. Current and Haynes (4) first described the in vitro cultivation of *C. parvum* by using human fetal lung cells and primary chicken kidney and porcine kidney cells. Approximately two dozen papers have subsequently described the development of *C. parvum* in cell culture (7). The parasite resides directly beneath the microvillous surface of epithelial cells in an extracytoplasmic location (8). While anatomical sites of infection usually involve the alimentary tract, other mucosal surfaces lined with epithelial cells (pulmonary system and uterine mucosa) are also permissive to *C. parvum* infection (3, 14, 15). The susceptibility of epithelial cells bearing prominent microvilli to infection by this parasite prompted us to investigate bovine fallopian tube epithelial (BFTE) cells as possible host cells. BFTE cells have especially long and thickened microvilli on their surfaces and are closely associated with the uterine mucosa.

The discovery of new anticryptosporidial compounds will be facilitated by the ability to precisely model the entire life cycle of *C. parvum* in cell culture. Moreover, in vitro culture will provide a useful tool for investigating interactions between parasites and host cells in the absence of intestinal bacteria. The objectives of the present study were to (i) develop a reproducible and quantitative BFTE cell culture system in which *C. parvum* can complete its entire life cycle, (ii) compare the growth and development of *C. parvum* in BFTE cells with growth and development in the widely used Madin-Darby canine kidney (MDCK) cells, and (iii) confirm that BFTE cell culture-derived oocysts are infective for mice.

C. parvum (bovine isolate) oocysts used in this study were originally donated by Harley Moon (U.S. Department of Agriculture, Ames, Iowa). The oocysts were isolated from calf manure as previously described (1), preserved in 2.5% potassium dichromate, and used within 3 months of purification. Oocysts were decontaminated by suspension in 20% (vol/vol) commercial bleach (1.05% sodium hypochlorite) on ice for 20 min. Oocysts were then washed three times in Hanks' balanced salt solution (HBSS) and once in RPMI 1640. Release of sporozoites was achieved by incubating oocysts in an excystation solution prepared by mixing 0.25% (wt/vol) trypsin and 0.75% (wt/vol) taurodeoxycholic acid (Sigma, St. Louis, Mo.) in HBSS. The suspension was incubated at 37°C for 45 min and microscopically observed to confirm sporozoite release. Excysted sporozoites were washed with RPMI 1640 without serum. Sporozoites were completely separated from intact oocysts and oocyst walls by passage through sterile polycarbonate filters (3-μm pore size; Millipore Corp., Bedford, Mass.) twice prior to inoculation of BFTE cell monolayers.

The growth and development of *C. parvum* in BFTE cells were compared with growth and development in MDCK cells.

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Primary BFTE cell cultures were prepared from bovine fallopian tubes (FT) collected from E. A. Miller & Sons Packing Company (Hyrum, Utah). The MDCK cells were obtained from the American Type Culture Collection (ATCC CCL 34). After fat was trimmed from the serosal surfaces and mucus was gently squeezed from the lumens, FT were decontaminated by being submerged in 70% ethanol for 30 s. The FT were then transferred to sterile culture petri dishes containing HBSS and washed twice. The BFTE cells were harvested either by flushing the FT with HBSS, using a 10-ml syringe equipped with a mouse feeding needle, or by opening the FT lengthwise with a scissors and scraping the mucosal surfaces. The BFTE cells were subsequently washed in HBSS by centrifugation at 200 × *g* for 10 min, planted in 75-cm^2 flasks containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, Utah), and cultured in a 5% CO_2 incubator at 37°C for 72 to 120 h. For routine cell passage, 10% FBS was used, whereas FBS was not used (growth medium) whenever parasites were used. The MDCK cells were cultured in the same medium and under identical conditions as BFTE cells. When the cell lines reached confluency, they were trypsinized, split, and planted onto coverslips positioned on the bottoms of individual wells in 24-well tissue culture plates. When cells reached 80% confluency, they were inoculated with either 10^5 oocysts or 4×10^5 sporozoites per well. Plates were then maintained at 37°C in a candle jar environment (17% O_2, 3% CO_2, 80% N_2) as previously described (16). Growth medium was changed in each well every 72 h. In those wells inoculated with oocysts, the medium was first changed at 24 h to remove any unexcysted oocysts.

Eight coverslips were removed at 5, 24, 48, 72, 96, and 120 h from both inoculated and uninoculated wells containing monolayers of BFTE and MDCK cells. The coverslips were washed in RPMI 1640, fixed in 100% methanol for 10 min, stained with Giemsa stain for 1 h, and washed three times with double-distilled water (ddH_2O). Coverslips were then mounted on glass slides and examined under oil immersion (1,000×), using bright-field microscopy. Parasites were enumerated by counting all developmental stages of *C. parvum* present in a single scan (67 fields) through the center of each coverslip. The data were statistically compared for significance, using analysis of variance (Fisher's protected least-significant-difference test) on a STATVIEW program (13).

Coverslips containing monolayers of BFTE cells were collected at 5, 24, 48, 72, 96, and 120 h from inoculated and uninoculated wells and prepared for scanning and transmission electron microscopy (SEM and TEM). A modified protocol described by Gabriel (9) was used to prepare the SEM samples. Briefly, BFTE cells on coverslips were fixed at room temperature with 2% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4) for 2 h and then incubated for 2 h in 2% tannic acid–2% glutaraldehyde. They were then washed overnight in PB, postfixed for 2 h in 2% OsO_4 in PB, and rinsed with ddH_2O for 3 h. The cells were incubated in 1% tricarbohydrazide in ddH_2O for 1 h, washed in ddH_2O for 2 h, immersed in 1% OsO_4 in ddH_2O for 2 h, washed in ddH_2O overnight, dehydrated in a graded series of ethanol, and dried in a critical point drying apparatus. Coverslips were then attached to stubs with adhesive, coated with gold palladium in a sputter coater, and observed with a Hitachi S-4000 field emission scanning electron microscope (Hitachi, Ltd., Tokyo, Japan).

A modified protocol reported by Griffiths (10) was used to prepare the TEM samples. The BFTE cells were gently scraped from the surfaces of coverslips, rinsed in RPMI 1640, and fixed with 3% glutaraldehyde in RPMI 1640 for 3 h. They were postfixed in 1% OsO_4 in RPMI 1640 for 1 h and again rinsed in RPMI 1640. The cells were dehydrated in a graded series of ethanol and embedded in Spurr resin (a low-viscosity epoxy resin embedding medium). Samples were then thin sectioned, stained, and mounted on a grid. Microexamination and photography were conducted by using a Zeiss 902CEM transmission electron microscope (Carl Zeiss, Inc., Thornwood, N.Y.).

To confirm the production of infective oocysts in cell culture, an immunosuppressed mouse model for cryptosporidiosis was used. Five groups of adult female C57BL/6N mice (6 weeks of age; each weighing 14 to 16 g) were purchased from Taconic (Germantown, N.Y.) and housed in the Laboratory Animal Research Center at Utah State University. Individually caged mice were immunosuppressed with dexamethasone phosphate (Sigma) provided ad libitum in drinking water at a dosage level of 12 μg/ml. At 120 h postinoculation, coverslips from individual 24-well plates were collected, and the surfaces were scraped and pooled for each plate. All mice in a group were gavaged on day 7 postimmunosuppression with an equal volume of the resulting cells, cell products, and parasites (plate product) harvested from a single plate. Group 1 (four mice) and group 2 (five mice) received the plate product from plates inoculated with oocysts and sporozoites, respectively. Group 3 (five mice) was treated the same as group 1 except that the plate product was first suspended in 70% ethanol for 10 min to kill all stages of *C. parvum* except the oocysts. Each mouse in group 4 (four mice) was inoculated with 10^4 oocysts from the same batch used to inoculate the BFTE cell monolayers. Each mouse in group 5 (seven mice) was inoculated with 10^4 intact oocysts which had been incubated for 120 h in wells from a plate containing only growth medium. Microscopic visualization revealed that approximately 90% of the oocysts used to inoculate the mice in group 5 had excysted and/or degenerated. Fecal samples were collected from recta each day from mice in all five groups and monitored for oocyst shedding, using an oocyst-specific monoclonal antibody-based indirect immunofluorescence assay (2). Mice were killed on day 14 postinoculation.

This study was approved by the Utah State University Institutional Animal Care and Use Committee and was done in the Utah State University Laboratory Animal Research Center, which is an American Association for the Accreditation of Laboratory Animal Care-accredited facility.

Successful infections were observed in BFTE cells inoculated with both oocysts and purified sporozoites. Parasites developed at the microvillous surface of BFTE cells in an intracellular but extracytoplasmic location. Moreover, multiple infections were common in individual cells, similar to those frequently observed in vivo.

The life cycle of *C. parvum* was monitored for 120 h in BFTE cell culture and is illustrated in Fig. 1 to 3. At 5 h postinoculation, asexual development was easily discernible, with trophozoites present within host cells (Fig. 1A and 2A). Meronts had developed by 24 h, approximately 75% of which were type I (with six or eight merozoites) (Fig. 1B, 2B, and 3A). Parasite development became much less synchronized after 24 h, and by 48 h, various intermediate stages in the life cycle of *C. parvum* were present. Approximately 20% of these intermediate stages were type II meronts (with four merozoites) (Fig. 2C and 3B). Sexual stages (gamonts) were first observed at 48 h but were particularly abundant by 72 h. Both microgamonts and macrogamonts were present (Fig. 2D and E). Newly formed oocysts were also first observed at 48 h but were more numerous in cell culture between 72 and 120 h (Fig. 2F). Various asexual stages of development persisted through 120 h.

Both BFTE and MDCK cells were permissive to infection by

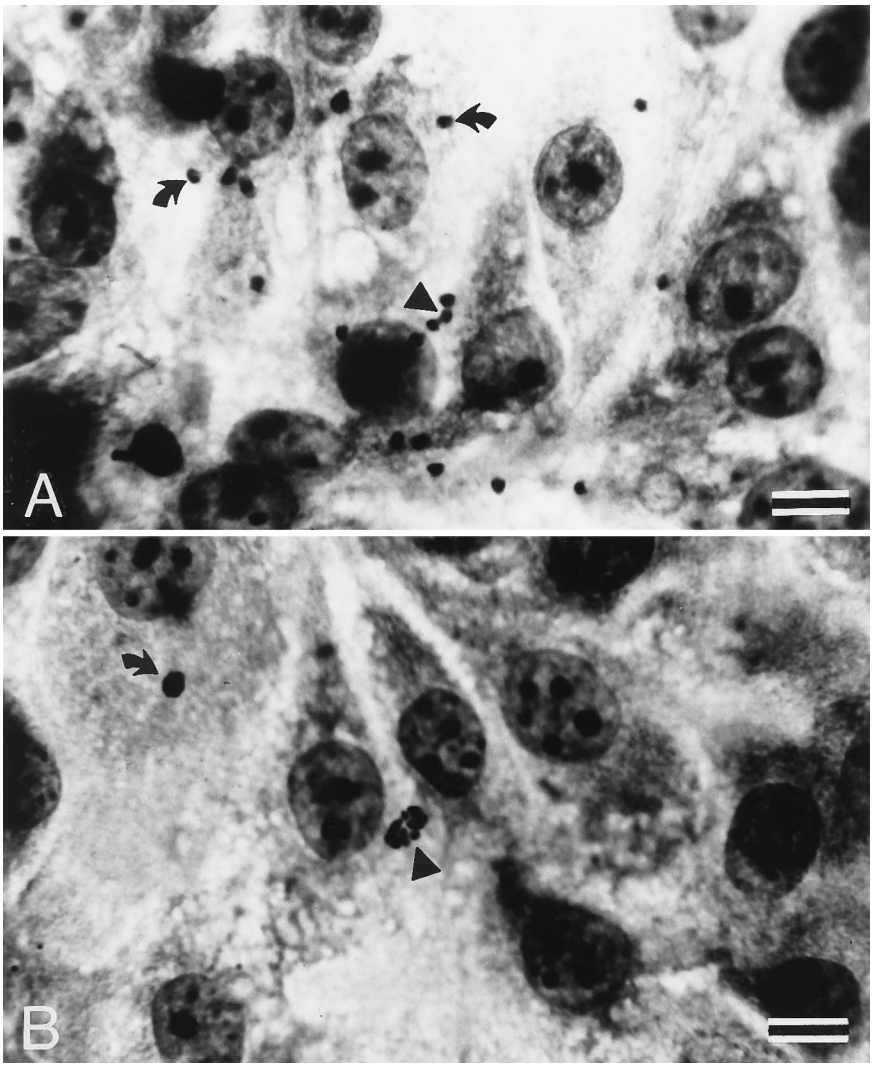

FIG. 1. Bright-field microscopy of BFTE cells infected with *C. parvum*. (A) Numerous trophozoites (arrows) within BFTE cells. Multiple infections of individual cells are apparent (arrowhead). (B) Early (arrow) and fully developed (arrowhead) type I meronts. Bars = 10 μm.

C. parvum (Fig. 4). While the patterns of parasite development in the two cell lines were similar, significantly ($P \leq 0.001$) more parasites were present in BFTE cells than in MDCK cells at each of the six sampling times. The largest number of parasites was present in both cell lines at 24 h. Infection rates for BFTE and MDCK cells were 39.4 and 4.6% at this time. Thereafter, the number of parasites decreased, except for a modest increase in BFTE cells at 120 h.

Oocysts produced in BFTE cell culture after 120 h postinoculation were infective for immunosuppressed adult C57BL/6N mice. All mice in groups 1 to 4 shed oocysts in their feces, with prepatent periods ranging from 2 to 6 days. Mice in groups 1 and 2 began shedding oocysts on days 5 (seven mice) and 6 (two mice). All mice in group 3 began shedding oocysts on day 4. Oocysts were detected in the feces of mice in group 4 on days 2 (one mouse) and 3 (three mice). Oocyst shedding continued until the mice were killed. Mice in group 5 did not shed oocysts.

This study is the first to demonstrate that *C. parvum* can complete its entire life cycle (from sporozoite to infective oocyst) in BFTE cells (Fig. 2). Consistent with results reported by Upton et al. (25), oocysts were superior to purified sporozoites as the infecting inoculum because they were simpler to use and less labor-intensive to prepare. Oocysts also produced consistent infection rates compared with those of the more fragile sporozoites. Infection of BFTE cells with *C. parvum* closely paralleled in vivo infections with regard to the chronology of parasite development. For example, oocysts are frequently passed in the feces of mice, dogs, pigs, lambs, and cattle by day 2 or 3 postinoculation (8, 26), although prepatent periods may vary among animal species. In BFTE cells, oocysts were first observed at 48 h but were most numerous between 72 and 120 h (Fig. 2F). Moreover, *C. parvum* developed at the microvillous surface of BFTE cells (Fig. 3) as it does in the intestinal epithelial cells of animals and humans (8, 24). Multiple parasitic infections were common in individual BFTE cells (Fig. 1A, 2A, and 3) as they are in vivo (20). Finally, long and thickened microvilli on BFTE cells (Fig. 3B), as well as the microvilli on intestinal epithelial cells, seem to be conducive to *C. parvum* infection, perhaps because the parasitic adhesins recognize receptor molecules on the microvilli which facilitate host cell attachment and invasion.

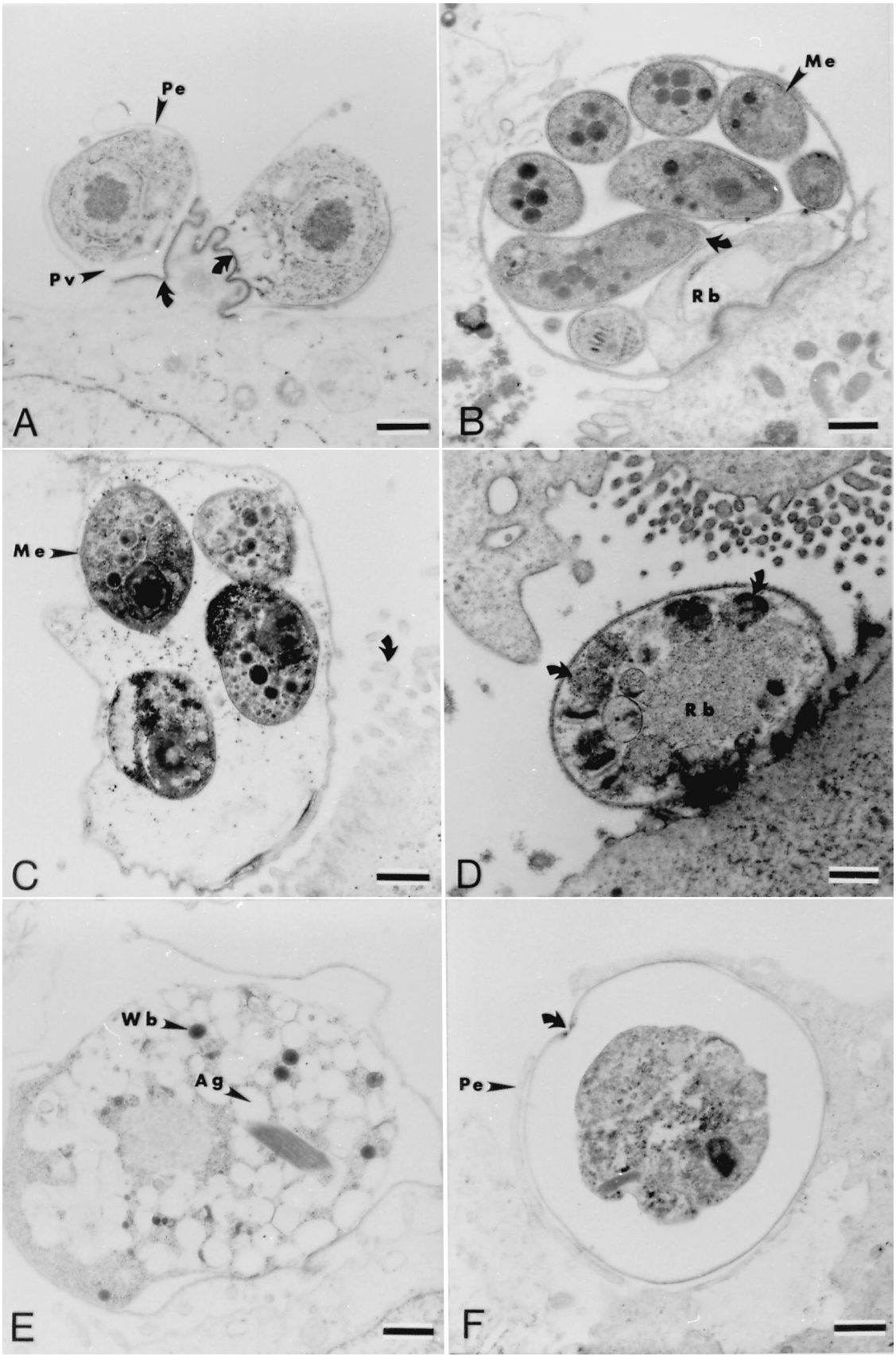
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FIG. 2. TEM of BFTE cells infected with *C. parvum*. (A) Two trophozoites with associated feeder organelles (arrows). Note the parasitophorous envelope and parasitophorous vacuole. (B) A type I meront containing eight distinct merozoites. One merozoite is in the process of budding (arrow) from the residual body. (C) A type II meront containing four merozoites. Microvilli (arrow) are present on the surface of BFTE cells. (D) A mature microgamont with portions of at least five microgametes (arrows) that have separated from the residual body. (E) A macrogamont containing wall-forming bodies and amylopectin granules. (F) An unsporulated oocyst almost fully encapsulated in the parasitophorous envelope. Note the suture in the oocyst wall (arrow). Pe, parasitophorous envelope; Pv, parasitophorous vacuole; Me, merozoite; Rb, residual body; Wb, wall-forming body; Ag, amylopectin granule. Bars = 0.6 μm.

Infecting BFTE cells with sporulated oocysts is a highly reproducible cultivation system that we have used during the past year to evaluate the anticryptosporidial activities of several pharmaceutical compounds. More parasites were consistently observed between 5 and 120 h in BFTE cells than in MDCK cells (Fig. 4). The latter cell line has been widely used for the in vitro culture of *C. parvum* (6, 11, 12, 22, 23). However, only the last two reports describe the development of sporulated oocysts in MDCK cells, and none confirmed oocyst infectivity for animals. Rosales et al. (22, 23) reported that *C. parvum* developed in the cytoplasm of MDCK cells near the host cell nucleus. This atypical site of development has not been reported in vivo and was never observed in BFTE cells wherein *C. parvum* developed extracytoplasmically at the microvillous surface. Rosales et al. (22) reported an infection rate of 3.6% in MDCK cells at 24 h, slightly lower than the 4.6% rate that we achieved in this cell line at the same time period but significantly ($P \leq 0.001$) less than the 39.4% rate achieved in BFTE cells. Although various asexual stages of development persisted for 120 h in BFTE cells, the number of parasites in MDCK and BFTE cells decreased between 24 and 96 h. In contrast to the continued decrease in MDCK cells, there was a modest increase in the number of parasites developing in BFTE cells between 96 and 120 h. This increase in *C. parvum*

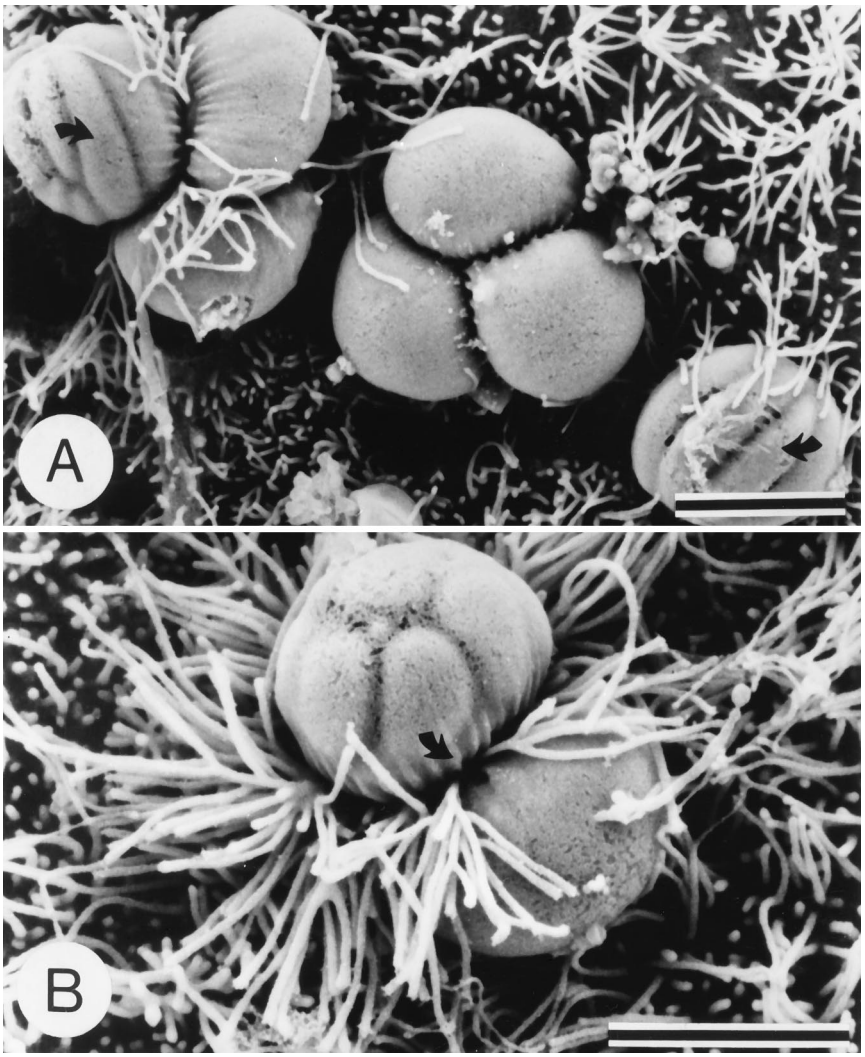

FIG. 3. SEM of BFTE cells infected with *C. parvum*. (A) Seven type I meronts in close association with micovilli which are characteristically found on the surfaces of BFTE cells. Individual merozoites (arrows) are discernible beneath the parasitophorous envelope of two mature meronts. (B) Two juxtaposed type II meronts. Again, merozoites can be seen beneath the parasitophorous envelope, which is fluted around its base (arrow). The surfaces of the parasitophorous envelopes appear to be disintegrating in preparation for merozoite release. Note the long and thickened microvilli. Bars = 3.0 μm.

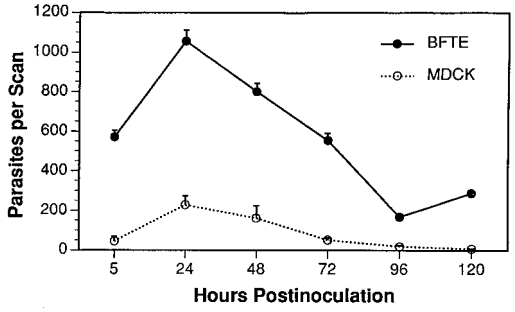

FIG. 4. Growth and development of *C. parvum* in BFTE and MDCK cells. The patterns of parasite development in the two cell lines inoculated with oocysts were similar between 5 and 120 h. However, significantly ($P \leq 0.001$) more parasites were present in BFTE cells than in MDCK cells at each of the six sampling times. Infection rates for BFTE and MDCK cells were 39.4 and 4.6% at 24 h. Values represent means ± standard errors.

is intriguing and unexplained. We speculate that autoinfection may result from cell culture-derived oocysts which subsequently excyst and release sporozoites, but such an event has not been observed in BFTE cell culture.

Oocysts produced in BFTE cell culture were infective for immunosuppressed adult female C57BL/6N mice. This mouse model for cryptosporidiosis was selected because it is comparatively inexpensive, is easy to maintain, can be consistently infected with *C. parvum*, and is subject to chronic infections (21, 26, 27). To our knowledge, only Current and Haynes (4) have demonstrated that cell culture-derived oocysts were infective for animals. We have previously shown that 100% of immunosuppressed adult C57BL/6N mice can be infected with as few as 10 oocysts per mouse, but at least 10^4 oocysts per mouse are required to produce a prepatent period of 3 days or less (28). This finding suggests that each of the mice in groups 1 to 3 received fewer than 10^4 oocysts, although the exact number is unknown. Oocysts incubated in growth medium for 120 h (group 5) were noninfective for mice, indicating that any residual oocysts from the cell culture inoculum (groups 1 and 3) were also noninfective. Clearly, the oocysts that produced patent infections in mice from groups 1 to 3 were cell culture derived.

This study describes the complete development of *C. parvum* (from sporozoite to infective oocyst) in BFTE cells. Photomicrographs of SEM and TEM were used to detail the ultrastructure of individual parasitic stages. Cultivation of *C. parvum* in BFTE cells will enable investigators to determine the biological, biochemical, and immunological relationships between the parasite and the host cell in the absence of other microbes commonly found in the intestinal tract. Cultivation of BFTE cells further provides a reproducible and quantitative system for testing the pharmacological activities of potentially prophylactic and therapeutic compounds.

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