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Infectivity and Neutralization of *Cryptosporidium parvum* Sporozoites

MICHAEL W. RIGGS* AND LANCE E. PERRYMAN

Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, Washington 99164-7040

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Cryptosporidiosis, a diarrheal disease of calves and humans caused by the coccidian parasite *Cryptosporidium parvum*, is terminated in hosts with normal immune systems. To assess the mechanisms of immunity in cryptosporidiosis, it is necessary to isolate and quantitate sporozoites, the infective stage of *Cryptosporidium* spp. Here we report the (i) separation of infective *C. parvum* oocysts from calf feces by ether extraction, sieving, and hypochlorite treatment; (ii) separation of viable *C. parvum* sporozoites from intact and excysted oocysts by anion-exchange chromatography; and (iii) quantitation of sporozoite infectivity in vivo by direct intraintestinal injection of isolated sporozoites in 7-day-old BALB/c mice. When isolated sporozoites were incubated with heat-inactivated immune bovine serum, 25 times the 50% infective dose for 7-day-old mice was completely neutralized. Sporozoites incubated with preimmune bovine serum were infectious for 7-day-old mice.

Cryptosporidium parvum is an intracellular coccidian parasite which infects the intestinal tract and a variety of extraintestinal epithelial sites (2, 6, 8, 12, 20–22, 27, 38, 40, 44). Cryptosporidiosis is characterized by self-limited diarrhea in immunocompetent animals and humans (1, 5, 10, 17, 18, 30, 39, 41, 43, 45), but persistent life-threatening diarrhea in immunodeficient hosts (10, 14, 37, 38). There are currently no immunization or specific therapeutic regimens for control of cryptosporidiosis (3, 15, 25). Control efforts are hampered further by the fact that oocysts are infective at the time of passage in feces and are not inactivated by commonly used disinfectants (4, 24).

Control of *C. parvum* infection by the immune system is indicated by the following observations. (i) The disease is self-limited in immunocompetent hosts, but is persistent in immunodeficient hosts; (ii) recovered immunocompetent calves and humans are resistant to reinfection (M. W. Riggs, unpublished observations); and (iii) immune anti-*Cryptosporidium* sp. bovine colostrum administered orally to a hypogammaglobulinemic child with persistent cryptosporidiosis resolved clinical signs associated with the disease and terminated oocyst shedding (42). For these reasons, investigations on immunologic approaches for control of the disease were initiated. The aim was to demonstrate that antibody against surface antigen(s) on the infective sporozoite stage of *C. parvum* neutralizes infectivity. In this report, we demonstrate that viable *C. parvum* sporozoites are infectious for mice when injected directly into the intestinal tract and that infectivity of sporozoites can be neutralized by immune bovine serum.

MATERIALS AND METHODS

The *C. parvum* isolate used for all experiments was obtained from H. Moon (National Animal Disease Center, Ames, Iowa). Pathogenicity of the isolate for calves and infectivity for mice have been established (16, 17). The isolate is also infectious for humans and causes diarrhea, nausea, abdominal cramps, and anorexia (M. W. Riggs, unpublished observations).

* Corresponding author.

Calf feces containing oocysts were stored in an equal volume of 2.5% potassium dichromate solution at 4°C. Oocyst viability was maintained by passage every 3 to 4 months in newborn Holstein calves obtained immediately after parturition and housed in isolation facilities. Prior to challenge, calves were verified to be free of *C. parvum* infection by repeated fecal examination for oocysts by using a modified Kinyoun acid-fast stain at 3 to 5 days of age (13).

Oocyst isolation. A modified ether extraction, sodium hypochlorite digestion procedure was used to separate oocysts from calf feces (31, 36). All steps were performed at 4°C. The fecal-potassium dichromate slurry was washed five times in 10 volumes of phosphate-buffered saline (PBS; 1,400 × *g*) and suspended in 5 volumes of 1% sodium bicarbonate solution and a quantity of ether equal to 1.2 times the original fecal pellet volume. The resulting mixture was vigorously shaken for 30 s. Following centrifugation at 1,400 × *g*, the ether-extracted fecal layer was removed, and the oocyst pellet was washed 3 times in PBS and sieved with a filter (pore size, 45 μm). Sieved oocysts were pelleted, suspended in 50 volumes of 1.75% sodium hypochlorite solution, placed in an ice water bath, and inverted every minute for 8 min. Finally, oocysts were washed 5 times in PBS.

To monitor for bacterial contaminants, the isolated oocyst preparation was cultured in brain heart infusion broth at 37°C for 7 days. The infectivity of isolated oocysts was established by oral inoculation of 10 5-day-old BALB/c mice with 10^5 oocysts. Mice were evaluated for *C. parvum* infection by histologic examination of the intestinal tracts 96 h postinoculation.

Sporozoite isolation. Isolated oocysts (5×10^7/ml) were suspended in Hanks balanced salt solution (HBSS) and incubated for 1.5 h in a shaking water bath at 37°C to release sporozoites. Sporozoites were stored in HBSS at 4°C before use.

Sporozoites were separated from intact and excysted oocysts in the postexcystation preparation by DEAE-cellulose anion-exchange chromatography at 4°C. DEAE-cellulose (DE-52; Whatman, Clifton, N.J.) was washed four times by suspending 1 g of DEAE-cellulose in 100 ml of column buffer (10.78 g of Na_2HPO_4 per liter, 0.62 g of $NaH_2PO_4 \cdot H_2O$ per liter, 3.4 g of NaCl per liter 10.0 g of

glucose per liter [ionic strength, 0.290; pH 8.0]), allowing the matrix to settle, and decanting the supernatant. The washed DEAE-cellulose was equilibrated overnight in column buffer, degassed for 10 min under vacuum, packed to a bed height of 1 cm in a disposable polypropylene chromatography column (diameter, 1 cm; Bio-Rad Laboratories, Richmond, Calif.), and rinsed with 20 ml of column buffer.

The excysted oocyst preparation was washed twice in column buffer (3,500 × *g*), centrifuged at 10 × *g* to remove coarse debris, and suspended in 20 ml of column buffer (5×10^6 sporozoites per ml) containing a 1-cm column height volume of DEAE-cellulose. The preparation was applied to the equilibrated column and allowed to flow through until the fluid level was within 1 mm of the top of the DEAE-cellulose bed. The matrix was then washed with 50 ml of column buffer. Sporozoites in the column eluate were washed twice in modified Eagle minimum essential medium with Earle salts (MEM) (Flow Laboratories, Inc., McLean, Va.), and stored in MEM at 4°C prior to use. Sporozoites and intact oocysts in the column eluate were quantitated with a hemacytometer.

Sporozoite infectivity assay. The viability of isolated sporozoites was determined with fluorescein diacetate and by fluorescence microscopy (23). This procedure was validated for viability determination of *C. parvum* by inclusion of Formalin-fixed and heat-killed (56°C, 30 min) control sporozoite preparations. By using sporozoites and oocysts isolated from calf feces which had been stored for 5 weeks in 2.5% potassium dichromate at 4°C, a 50% mouse infective dose (MID_{50}) was calculated (32). Groups of five 7-day-old BALB/c mice were challenged with 100, 1,000, 10,000, 100,000, or 1,000,000 isolated sporozoites per mouse or with 200, 2,000, 10,000, or 200,000 isolated oocysts per mouse by intraintestinal injection. Intestinal tracts were examined histologically 96 h postchallenge for *C. parvum* infection, as described below.

Immune serum. Isolated oocysts were excysted as described above. The postexcystation mixture was washed twice in HBSS at 4°C and mixed with monophosphoryl lipid A–trehalose dimycolate emulsion (Ribi Immunochem Research Inc., Hamilton, Mont.). A 14-month-old Holstein heifer was immunized by intramuscular injection of 1 ml of emulsion containing 1.5×10^8 sporozoites and boosted intramuscularly 1 month later with 3×10^8 viable sporozoites in 2 ml of incomplete Freund adjuvant. Intramuscular boosting with 3×10^8 to 1×10^9 viable sporozoites in HBSS was then performed every 2 weeks for 10 weeks (five injections).

The anti-sporozoite titer of serum obtained from the heifer prior to immunization and 2 weeks following the last boost was determined by indirect immunofluorescence. All steps were performed at 4°C by using viable sporozoites (5×10^7/ml) in PBS with 0.5% (wt/vol) bovine serum albumin (PBS-BSA; Sigma Chemical Co., St. Louis, Mo.).

The assay was performed in 96-well V-bottom microtitration plates (Flow Laboratories). All dilutions were made with PBS-BSA. A total of 50 μl of diluted, heat-inactivated (56°C, 30 min) serum and 50 μl of sporozoite preparation per well were incubated for 30 min and then washed four times with 200 μl of PBS-BSA per well (3,500 × *g*). A total of 50 μl of fluorescein-conjugated affinity-purified rabbit anti-bovine immunoglobulin G (Jackson Immunoresearch Laboratories, Avondale, Pa.) diluted 1:30 was then mixed with the sporozoite preparation in each well. Following a 30-min incubation, sporozoites were washed 4 times with PBS-BSA and stored at 4°C before examination by fluorescence microscopy.

The viability of a control sporozoite preparation subjected to washing procedures as described above was determined with fluorescein diacetate.

Sporozoite neutralization assay. The sporozoite neutralization assay was performed in a 96-well round-bottom tissue culture plate (Flow Laboratories). The isolated sporozoite suspension (15 μl) in MEM was added to each well, mixed with an equal volume of MEM or heat-inactivated bovine serum diluted 1:5 with MEM (final serum dilution, 1:10), and incubated at 37°C for 30 min in a 5% CO_2 atmosphere. The bovine serum samples used were obtained from the same heifer prior to immunization with sporozoites and 2 weeks following the final sporozoite boost. The viability of isolated sporozoites before and after incubation with sera was determined with fluorescein diacetate.

Following incubation, sporozoites were suspended by drawing up and then gently expulsing the well contents 5 times with a 25-μl syringe (Hamilton Co., Reno, Nev.). A total of 25 μl from each well containing 2×10^5 sporozoites was then injected intraintestinally into 7-day-old specific-pathogen-free BALB/c mice. Uninfected control mice received 25 μl of MEM. Groups of mice were injected with sporozoites which had been incubated with preimmunization serum, postimmunization serum, or MEM. A 25-μl syringe fitted with a 4-cm length of polyethylene tubing (outer diameter, 0.61 mm; Clay Adams Co., Parsippany, N.J.) was used for intraintestinal injection. The tubing was inserted 2 cm into the colon through the anus prior to injection. Following injection, mice were maintained in a 37°C incubator. Removable rubber patches were used to prevent defecation for 5 h postinjection before mice were returned to their dams. Each group of mice was obtained from one litter and caged separately in containers fitted with filter tops.

Mice were euthanized with ether 96 h postchallenge. The entire intestinal tract was collected immediately after euthanasia, cut into 2.5-cm sections, and fixed longitudinally in buffered neutral Formalin. Hematoxylin-eosin-stained longitudinal sections representing the entire length of the intestinal tract from each mouse were prepared, assigned randomly selected numerical codes, and examined histologically without knowledge of treatment group. Scores of 0, 1, 2, or 3 representing the density of organisms per unit length of intestinal mucosa were assigned for duodenum, jejunum, ileum, cecum, and colon (0, absence of infection; 1, 1 to 33% of mucosa parasitized; 2, 34 to 66% of mucosa parasitized; 3, greater than 67% of mucosa parasitized). Cumulative scores that included each intestinal region were calculated for each mouse.

RESULTS

Oocyst isolation. Ether extraction separated 80 to 90% of the starting fecal volume from oocysts, and sieving effectively removed the remaining coarse debris. Sodium hypochlorite treatment dissolved residual microscopic debris and inactivated the microbial contaminants that were detectable by culture in brain heart infusion broth. This three-step procedure yielded an oocyst preparation that was suitable for accurate quantitation and excystation to obtain sporozoites (Fig. 1). Up to 7×10^9 oocysts per liter of feces were isolated from experimentally infected calves at the peak of oocyst shedding. Infectivity of isolated oocysts was established by (i) microscopic evidence of *C. parvum* intestinal infection in 10 of 10 mice challenged per os with 10^5

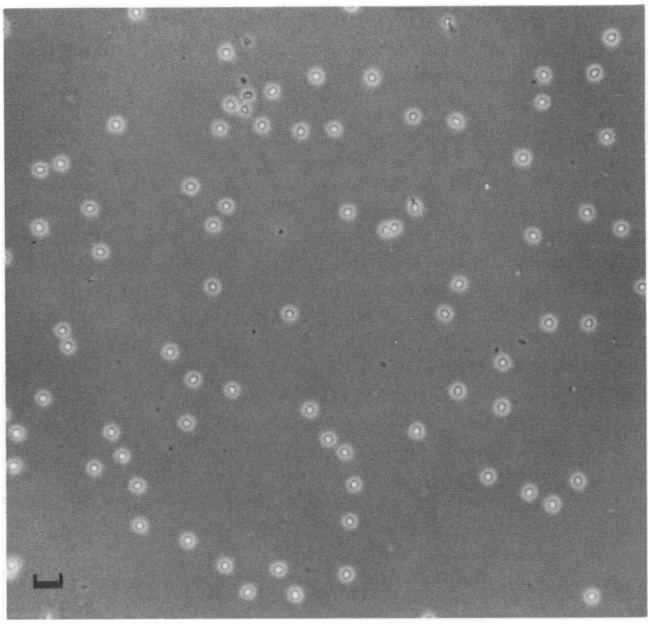

FIG. 1. Photomicrograph of *C. parvum* oocysts isolated from calf feces by ether extraction, sieving, and hypochlorite treatment. Bar, 20 μm.

oocysts and (ii) diarrhea, depression, and anorexia in 3 of 3 4- to 7-day-old calves challenged by per os administration of 2×10^8 to 3×10^8 oocysts, with fecal oocyst counts indicating amplification of the challenge inoculum.

Sporozoite isolation. In preliminary experiments, sporozoites, intact oocysts, and excysted oocysts bound to DEAE-cellulose equilibrated in PBS (ionic strength, 0.145; pH 8). Sporozoites were selectively eluted from the matrix by an increasing stepwise ionic strength gradient. PBS (ionic strength, 0.290; pH 8) gave maximal elution of sporozoites and minimal elution of intact oocysts. Maximal elution of intact oocysts occurred with buffer (ionic strength, 0.506; pH 8). Column buffer (ionic strength, 0.290; pH 8) was subsequently used for isolating sporozoites.

Mechanical clogging of the matrix decreased flow rates and resulted in total sporozoite recoveries of 10 to 20%. Sporozoite recovery was increased by suspending 50% of the final desired bed volume of DEAE-cellulose in the precolumn sporozoite preparation, to allow the DEAE-cellulose to pack gradually as the loading volume containing DEAE-cellulose, sporozoites, intact oocysts, and excysted oocysts passed through the column. Sporozoite recovery was increased further by temporarily interrupting the flow through the column and gently stirring the upper 50% of the bed volume to free the clogged material. Under optimized conditions, up to 90% of the sporozoites applied to the

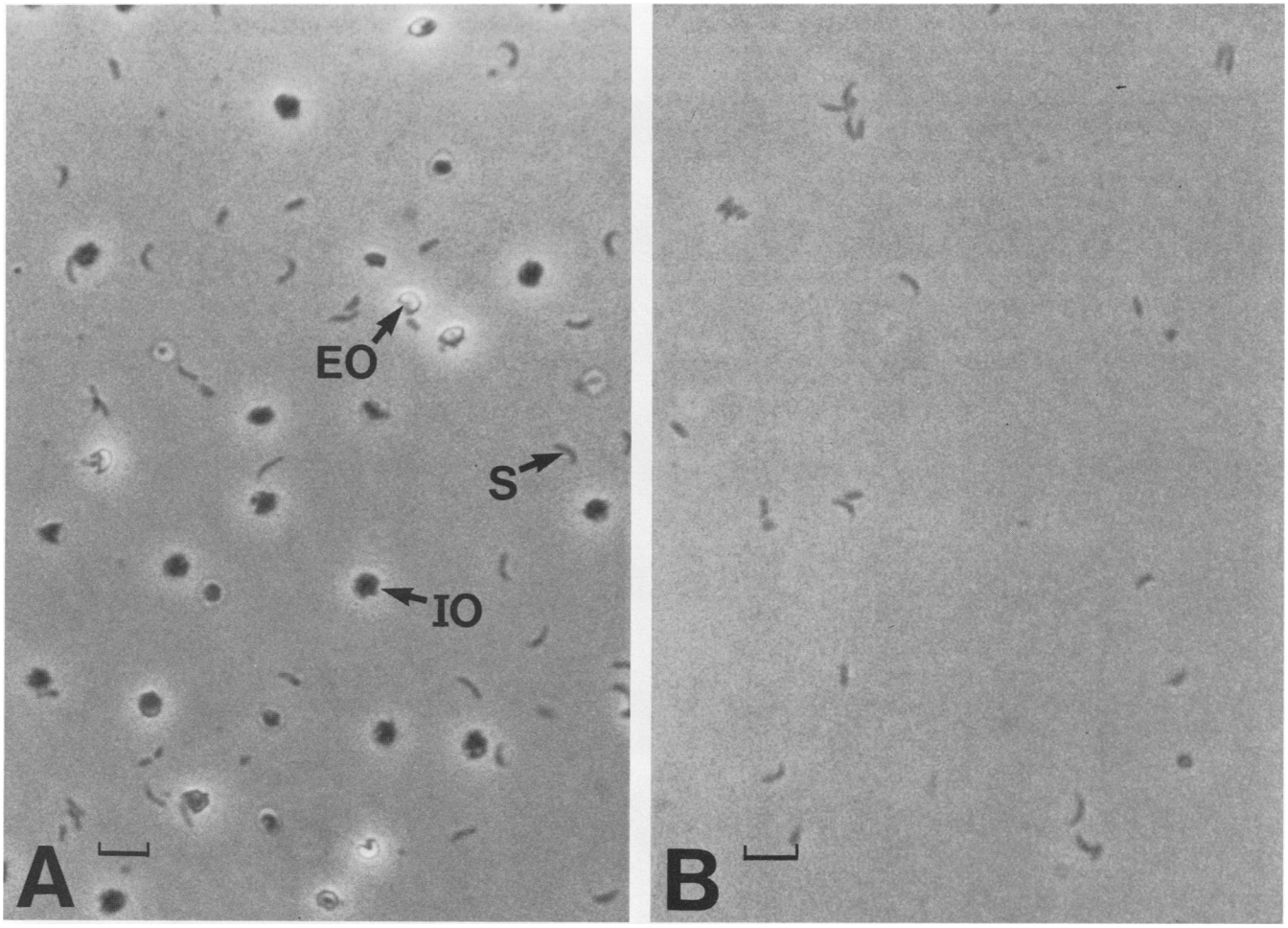

FIG. 2. Photomicrographs of *C. parvum*. (A) Postexcystation mixture of sporozoites (S), intact oocysts (IO), and excysted oocysts (EO). (B) Sporozoites isolated from the postexcystation mixture by DEAE-cellulose anion-exchange chromatography. Bar, 10 μm.

TABLE 1. Neutralization of *C. parvum* sporozoites by immune bovine serum

Expt and inoculum[a]	No. infected/ no. examined	Mean infection score ± SD[b]
Expt 1		
Medium	0/2	0
Sp + medium	7/7	7.3 ± 0.5
Sp + preimmunization bovine serum (1:10)	7/7	6.1 ± 0.7
Sp + postimmunization bovine serum (1:10)	0/7	0
Expt 2		
Medium	0/2	0
Sp + medium	2/2	3.5
Sp + preimmunization bovine serum (1:10)	5/5	4.6 ± 1.6
Sp + postimmunization bovine serum (1:10)	0/7	0

[a] Sporozoites were incubated in vitro with MEM or bovine serum (37°C, 5% CO_2, 30 min) before 7-day-old BALB/c mice were inoculated. Sp, 2×10^5 sporozoites.
[b] Determined 96 h postinoculation.

column were recovered, and contamination with intact oocysts ([number of intact oocysts/number sporozoites] × 100) was reduced from up to 73% in precolumn preparations to 1.7% or less in postcolumn preparations. Sporozoites used for determination of the MID_{50} had intact oocyst contamination of 0.06%. The viability of postcolumn and heat or Formalin-inactivated sporozoites was 90 to 95% and 0%, respectively. Representative pre- and postcolumn sporozoite preparations are shown in Fig. 2.

In preliminary experiments 10^5 isolated sporozoites and 1,200 contaminating intact oocysts did not infect four of six 8-day-old BALB/c mice when administered by gastric intubation. In two of six mice that became infected, infection scores were comparable to those of four age-matched control mice which were challenged with 1,200 intact oocysts by gastric intubation. This observation indicates that the infectivity of sporozoites is eliminated or substantially reduced when sporozoites are administered by gastric intubation. Attempts were then made to infect mice with sporozoites by intraintestinal injection. In preliminary experiments, 25 μl of PBS colored with blue dye injected intraintestinally through the anus into 7-day-old mice reproducibly spread to the entire colon, cecum, and terminal 2 cm of the small intestine. DEAE-cellulose-isolated sporozoites were infectious for 7-day-old mice by the intraintestinal route. The MID_{50} for sporozoites and oocysts was approximately 8,000 and 1,000, respectively.

Neutralization of sporozoites by immune serum. By live indirect immunofluorescence, pre- and postimmunization bovine sera had anti-sporozoite titers of 200 and 10,000, respectively. Prior to incubation with bovine sera, the viability of the isolated sporozoite preparation was 90%. After incubation with pre- or postimmunization bovine serum,

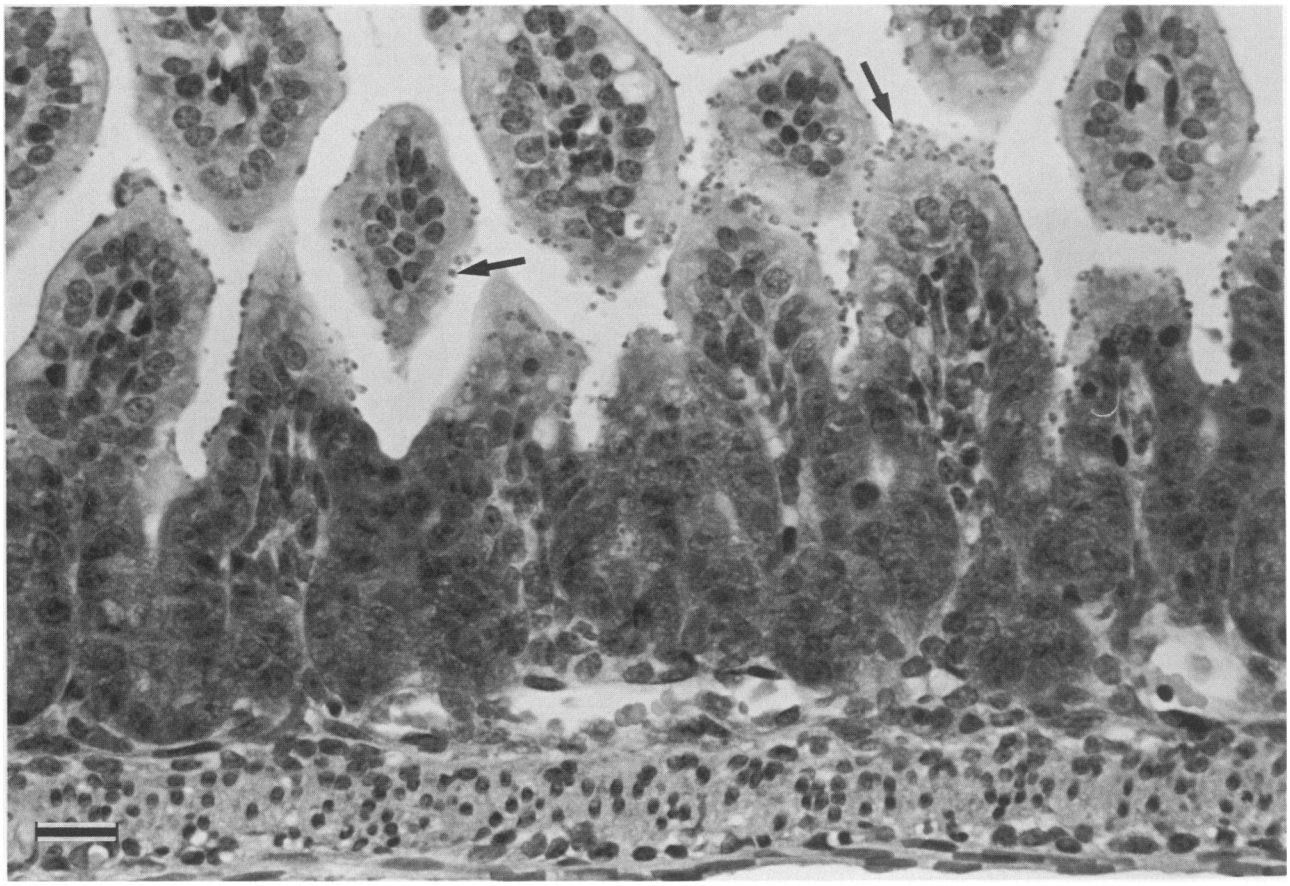

FIG. 3. Photomicrograph of ileum from a mouse challenged with MEM-treated sporozoites. Cumulative infection score of 8. Bar, 25 μm.

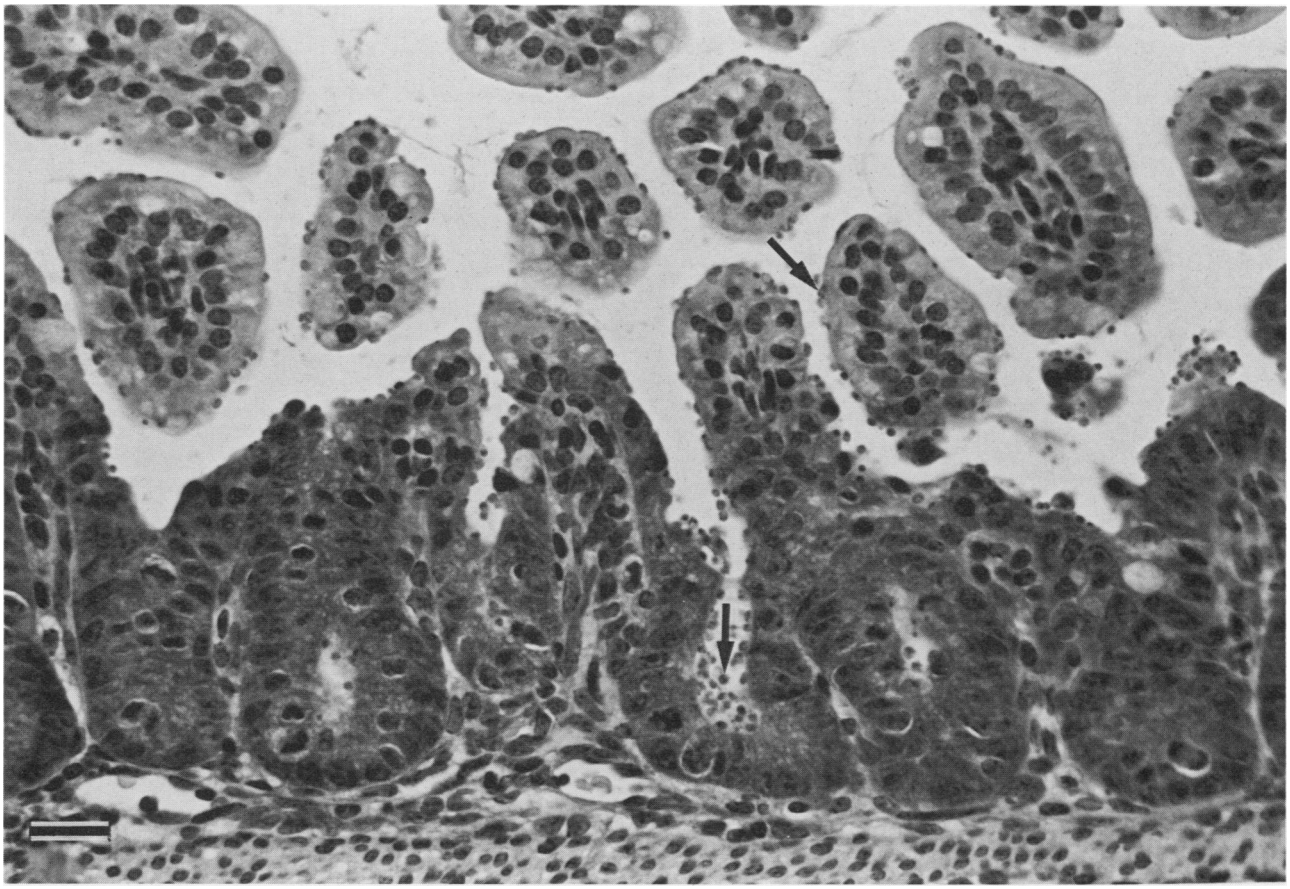

FIG. 4. Photomicrograph of ileum from a mouse challenged with preimmunization bovine serum-treated sporozoites. Cumulative infection score of 7. Note the similar numbers of cryptosporidia (arrows) infecting mucosa in Fig. 3 and 4. Bar, 25 μm.

sporozoite viability was 86 or 82%, respectively. Sporozoite agglutination in pre- and postimmunization bovine serum-treated sporozoite preparations was minimal prior to intraintestinal injection. Contamination of the sporozoite preparation with intact oocysts was 1.7%. Heat-inactivated postimmunization bovine serum completely neutralized 25 times the MID_{50} of *C. parvum* sporozoites (Table 1). Mice inoculated with sporozoites treated with preimmunization serum from the same heifer had infection scores similar to those of mice inoculated with MEM-treated sporozoites (Table 1). Representative sections of ileum from each challenge group demonstrate these findings (Fig. 3, 4, and 5). In infected mice, ileum, cecum, and colon were consistently parasitized. Jejunal infection was seldom observed, and neither gastric nor duodenal infection was observed. This same anatomic pattern of infection was observed in mice which were inoculated orally with 2×10^5 isolated oocysts.

DISCUSSION

Ether extraction, sieving, and hypochlorite treatment is an effective, technically simple method for large-scale separation of *C. parvum* oocysts from calf feces. Because isolated oocysts are infectious and can be accurately quantitated once they are separated from fecal matter, this method should be useful in obtaining oocysts for challenge studies to assess prophylactic regimens against cryptosporidiosis. Furthermore, an oocyst fraction which is largely free of fecal contamination facilitates sporozoite isolation. Preservation of surface antigens on isolated oocysts is suggested by the observation that two monoclonal antibodies and mouse anti-oocyst serum that is reactive by indirect immunofluorescence with intact oocysts in fresh calf feces have the same pattern of reactivity with intact oocysts isolated by ether extraction and hypochlorite treatment (unpublished observations).

It was essential in our strategy for detecting neutralization to have isolated, infectious sporozoites because intact oocyst contamination could result in a false-negative test result. Anion-exchange chromatography has been useful for separating several protozoa from contaminating host or parasite material (19, 26, 34). The data presented here indicate that the method is also effective for separating *C. parvum* sporozoites from intact and excysted oocysts. Isolated sporozoites retain surface antigens that are recognized by monoclonal antibodies and mouse anti-sporozoite serum and should be suitable for immunological and biological studies (unpublished observations).

The observation that infectivity was eliminated or reduced when viable *C. parvum* sporozoites were administered by gastric intubation presumably reflects inactivation in the gastric environment. Others have observed that *Eimeria* sp. sporozoites are not infectious when administered orally to chickens, but are infectious when injected directly into the intestinal tract (7, 28, 29, 33).

The data indicate that *C. parvum* sporozoites isolated by

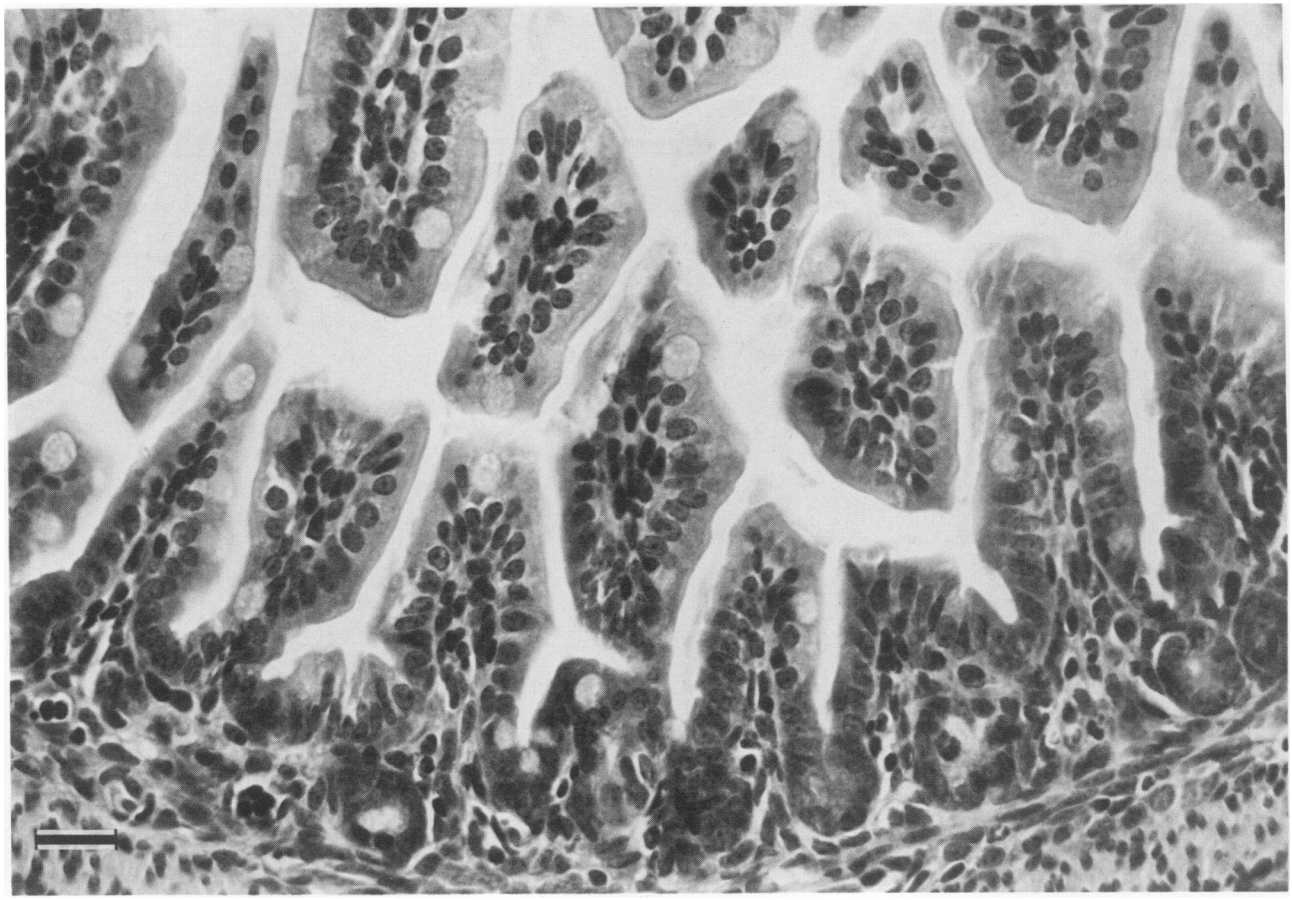

FIG. 5. Photomicrograph of ileum from a mouse challenged with immune bovine serum-treated sporozoites. Note the absence of infection with *C. parvum* (cumulative infection score of 0). Bar, 25 μm.

anion-exchange chromatography are infectious for 7-day-old BALB/c mice when injected directly into the intestinal tract. By this method, it was possible to determine a MID_{50} for isolated sporozoites and oocysts. The intraintestinal MID_{50} determined for ether-extracted, hypochlorite-treated oocysts (1,000 oocysts) is similar to the oral MID_{50} reported by other investigators who used untreated oocysts (1,200 oocysts) or 0.5% hypochlorite-treated oocysts (400 oocysts) obtained from calf feces (11, 35). This suggests that isolation procedures do not appreciably lower oocyst infectivity.

Because surface structures on *C. parvum* sporozoites are probably involved in the infection process and are possible targets for protective immune responses, cattle were immunized with viable, infectious sporozoites to obtain immune serum. Immune bovine serum neutralized 25 MID_{50}s of isolated sporozoites, as well as any sporozoites released intraintestinally from 3.4 MID_{50}s of contaminating oocysts. Antibody against sporozoite surface epitopes may be important for neutralization because the anti-sporozoite titer by live indirect immunofluorescence in the immune serum was 10,000, representing a 50-fold titer increase in the preimmunization serum which lacked neutralizing activity. Neutralization was not dependent on the presence of bovine complement because heat-inactivated serum had neutralizing activity.

The sporozoite infectivity assay was terminated at 96 h postchallenge. This is considered a sufficient period of time for histologic manifestation of infection, as evidenced by widespread intestinal infection in the control groups, and knowledge that completion of the entire life cycle in oocyst-challenged mice takes only 72 h (9). Because longitudinal sections representing the entire length of the small and large intestinal tracts were examined, our definition of neutralization discounts artifacts due to shifting of the anatomic site of infection within the intestinal tract or concentration of infection at one or more easily overlooked intestinal sites.

In this report, we have presented methods for isolating infectious *C. parvum* oocysts and sporozoites and for quantitating their infectivity by an in vivo assay. This assay should be useful for the evaluation of various anti-sporozoite treatments, including monoclonal antibodies and chemotherapeutic agents, and for quantitating merozoite infectivity. Neutralization studies with anti-sporozoite monoclonal antibodies are under way in our laboratory. Our observation that immune bovine serum neutralizes sporozoite infectivity demonstrates that cattle are capable of immunologic response to neutralization-sensitive epitopes on the infective stage of *C. parvum* and satisfies one criterion for successful immunoprophylaxis in cattle. This immune serum will be useful in defining neutralization-sensitive sporozoite antigens and may have limited application for the treatment of cryptosporidiosis in immunocompromised patients.

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