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Identification of a 15-Kilodalton Surface Glycoprotein on Sporozoites of *Cryptosporidium parvum*†

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An immunoglobulin A monoclonal antibody (MAb5C3) was developed against a 15-kDa surface glycoprotein (GP15) of *Cryptosporidium parvum* sporozoites. Indirect immunofluorescence and colloidal gold immunoelectron microscopy revealed that the antibody reacted with both the sporozoite and merozoite surface plasma membranes. On Western immunoblots, MAb5C3 binding was found to be strongly inhibited when 200 mM *N*-acetylglucosamine was used as a competing sugar. *N*-Acetylgalactosamine inhibited binding of the antibody only slightly, whereas glucose, mannose, and galactose failed to inhibit binding. MAb5C3 was found to recognize a similar 15-kDa epitope associated with a *Cryptosporidium* sp. isolated from guinea pigs. However, MAb5C3 failed to react with any proteins or glycoproteins associated with *C. baileyi* from chickens, *Cryptosporidium* sp. (= bovine *C. muris*) from cattle, *C. serpentis* from a rat snake, bradyzoites of *Besnoitia darlingi* from an opossum, sporozoite/oocyst extracts of *Caryospora bigenetica* from an eastern diamondback rattlesnake, sporozoites of *Eimeria nieschulzi* and *E. papillata* from rats and mice, or tachyzoites of *Toxoplasma gondii* (RH strain). When hybridoma supernatants containing MAb5C3 were administered orally to suckling mice experimentally infected with *C. parvum*, a 75% reduction in developmental stages was seen histologically at 72 h postinfection and a 67.5% reduction in mean oocyst output was found at 6 days postinfection.

Infections with *Cryptosporidium parvum* are generally self-limiting in immunocompetent individuals, manifested by moderate to severe diarrhea, weight loss, abdominal cramping, headache, and nausea (8). In immunocompromised individuals, however, infections with *C. parvum* may become persistent. The extent of fluid and electrolyte imbalance becomes so severe as to result in a life-threatening situation.

Due to difficulties associated with purification of *Cryptosporidium* spp. and because in vitro and in vivo models have not yet been perfected for these parasites, little is known about the immunobiology or molecular biology of *Cryptosporidium* spp. Monoclonal antibodies (MAbs) have recently been used as probes against *C. parvum* oocysts, sporozoites, and merozoites to identify specific proteins (1, 3, 20, 23). This article describes some characteristics of one such antibody, which recognizes a 15-kDa surface glycoprotein of *C. parvum* sporozoites.

MATERIALS AND METHODS

Parasites. Details on passaging, collecting, and purifying oocysts of *C. parvum* (KSU-1 isolate) have been described previously (27, 30, 31). Oocysts were further purified on CsCl gradients (14, 30) and frozen at −75°C with 10 μg/ml each of the protease inhibitors aprotinin, leupeptin, phenylmethylsulfonyl fluoride, *N*-*p*-tosyl-L-lysine chloromethyl ketone, and L-1-tosylamido-2-phenylethylchoromethyl ketone.

Oocysts of *C. baileyi* were cultivated and harvested from chicken embryos, *Gallus gallus*, as described previously (15). Oocysts of *Cryptosporidium* sp. (*C. muris* [31, 40]) were obtained from the feces of a naturally infected steer, *Bos taurus*, near Caldwell, Idaho (31). *C. serpentis* was obtained from the feces of a naturally infected yellow rat snake, *Elaphe obsoleta quadrivittata*, housed at the Houston Zoological Gardens. Oocysts of a *Cryptosporidium* sp. were recovered from the feces of experimentally infected guinea pigs, *Cavia porcellus* [Crl: (HA)BR] as described previously (5). Oocysts of all isolates were partially separated from fecal or cellular debris by sucrose flotation, purified further on CsCl gradients, and frozen at −75°C with protease inhibitors as described above for *C. parvum*.

Sporozoites of *Eimeria nieschulzi* and *E. papillata* are laboratory strains propagated in rats and mice, respectively. Details on purification of oocysts from feces have been published previously (4, 25, 26, 28, 29). Oocysts were stored at 4°C and treated prior to use for 14 h with 100% Clorox at 4°C (13). Oocysts were washed three times with sterile distilled water and twice with sterile phosphate-buffered saline (PBS) before removal of oocyst walls, excystation, and purification of sporozoites through a nylon wool column (26, 29). Sporozoites were washed three times in PBS and frozen at −75°C with protease inhibitors (see above) until use.

Oocysts of *Caryospora bigenetica* were obtained from the feces of a naturally infected eastern diamondback rattle-

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snake, *Crotalus adamanteus*. Collection and purification of oocysts of this isolate have been reported previously (39). Oocysts were further purified on CsCl gradients and frozen whole at −75°C with protease inhibitors as described above for *C. parvum*.

Cysts of *Besnoitia darlingi* were dissected from the viscera of a naturally infected opossum, *Didelphis marsupialis*, submitted to the Kansas State University College of Veterinary Medicine for necropsy. Bradyzoites were liberated from cysts by mechanically rupturing cysts in PBS with forceps. Bradyzoites were counted by hemacytometer and frozen at −75°C with protease inhibitors (see above).

Tachyzoites of *Toxoplasma gondii* (RH strain) were harvested from the peritoneal cavities of BALB/c mice, *Mus musculus*, inoculated with tachyzoites intraperitoneally 4 days previously (7). Tachyzoites in PBS were passed through a 27-gauge needle five times to rupture host cells and syringe passaged through 5-μm-pore-size Nuclepore filters. Tachyzoites were frozen at −75°C with protease inhibitors (see above).

Hybridoma production. BALB/c mice were immunized with CsCl-purified oocysts of *C. parvum* subjected to three cycles of freezing and thawing. All immunizations were subcutaneous, with an equivalent of 10^6 oocysts. The first immunization was with Freund's complete adjuvant, whereas the second was 2 weeks later with Freund's incomplete adjuvant. The third immunization at week 4 was without adjuvant. Spleens were removed, B-lymphocytes were fused with FOX.NY myeloma cells, and the cells were grown in Dulbecco's modified Eagle's medium (DMEM), as described before (6). Supernatants were tested for anti-*Cryptosporidium* antibodies by enzyme-linked immunosorbent assay (ELISA) (34, 37). Hybridomas producing anti-*C. parvum* antibody were cloned by limiting dilution and further assessed for reactivity by Western immunoblot analysis (see below).

Antibody characterization. Antibody isotypes were determined by a commercially available ELISA isotype kit (Hyclone Inc.). Antibody levels in supernatants were quantitated by radial immunodiffusion (24).

Electrophoresis and immunoblotting. Methods for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of coccidia have been described previously (27, 29–31). Following electrophoresis on a Bio-Rad mini-gel unit with 12% acrylamide gels, oocyst and sporozoite proteins were blotted onto nitrocellulose by using a Genie blotter (Idea Scientific, Corvallis, Ore.). Following transfer, blots were stained with Ponceau S (Sigma) to visually check for successful transfer of parasite proteins. Blots were then blocked for 1 h in 5% (wt/vol) powdered nonfat dry milk in PBS and then incubated with hybridoma supernatant for 2 h. Control lanes were incubated with supernatant from parental myeloma cells (DMEM). After being washed for 30 min in blocking solution with fresh changes at 5-min intervals, blots were probed for 1 h with a 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse polyvalent immunoglobulin. After being washed in blocking solution for 30 min as above, followed by a 5-min rinse in PBS, blots were developed with 4-chloro-1-naphthol. For competition experiments, 200 mM competing carbohydrate was added along with the hybridoma supernatant. Control lanes for the carbohydrate binding assays included 200 mM carbohydrate in DMEM in the absence of hybridoma supernatant.

Various species of *Cryptosporidium* and other coccidia were used to ascertain the cross-reactivity of MAb5C3. Pilot studies (not shown) were run to determine appropriate numbers of each parasite per lane. Equivalents of 1.25×10^6 *C. parvum*, *C. serpentis*, and *Cryptosporidium* sp. (guinea pig) oocysts were used, 7.5×10^5 *Cryptosporidium* sp. (= bovine *C. muris*) oocysts, 8.5×10^5 *C. baileyi* oocysts, 4.0×10^5 *Besnoitia darlingi* bradyzoites, 1.0×10^5 *Caryospora bigenetica* oocysts, 6.0×10^4 *Eimeria nieschulzi* and *E. papillata* sporozoites, and 4.0×10^5 *Toxoplasma gondii* tachyzoites were used.

In vitro cultivation. In order to determine whether MAb5C3 cross-reacted with stages other than the sporozoite, asexual and sexual stages of *C. parvum* were cultivated in vitro. Madin-Darby bovine kidney (MDBK) cells were first grown to 50% confluency on 22-mm^2 glass coverslips in six-well culture plates (Costar). The culture medium consisted of complete RPMI 1640 supplemented with 10% fetal bovine serum, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin B (Fungizone) per ml. Cesium chloride gradient-purified oocysts of 1-month-old *C. parvum* were surface sterilized in 5% (vol/vol) Clorox on ice and washed three times in sterile distilled water and twice in sterile PBS. Intact oocysts were added directly without excystation to the MDBK cells at a concentration of 5.0×10^6 oocysts per well. Parasites and cells were incubated for 72 h at 37°C in dessicator jars modified as candle jars (28). Coverslips were then washed three times in PBS, fixed in 10% neutral buffered Formalin, processed, and examined for epifluorescence as described below.

Fluorescent-antibody assays. Cesium chloride gradient-purified oocysts of *C. parvum* were treated with 10% Clorox on ice and washed twice in distilled water and then three times in PBS. Oocysts were incubated at 37°C for 1 h in 100% PBS to partially excyst oocysts. The excystation mixture, containing free sporozoites and intact and empty oocysts, was placed on ovalbumin-coated microscope slides and air dried at room temperature. Slides were stored at 4°C in the presence of $CaCl_2$ as a dessicant until used.

Prior to use, slides were rehydrated in PBS and incubated with hybridoma supernatant for 1 h in a humidified chamber. After incubation, slides were washed in PBS and then incubated with a 1:100 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse polyvalent immunoglobulin (Cappel-Organon/Teknika) in PBS for 1 h. Slides were then washed in PBS, counterstained with Evan's blue, coverslipped, and observed and photographed with an Olympus XEL epifluorescent microscope.

Immunoelectron microscopy. Ileum containing developmental stages of *C. parvum* was collected 3 and 7 days postinfection (PI) from suckling mice experimentally infected per os at 4 to 5 days of age with 10^4 oocysts. Free sporozoites of *C. parvum* were prepared by excysting CsCl gradient-purified oocysts (see above) and passing the mixture through a DE-52 anion-exchange column (22). The enriched sporozoite preparation, which also contained some intact oocysts, as well as ileum samples was fixed for 5 to 10 min on ice in 0.5% glutaraldehyde–4% paraformaldehyde in 0.1 M cacodylate buffer, dehydrated, and embedded in LR White resin as described previously (2). Thin sections were processed with supernatants containing MAb5C3 as the primary antibody and a goat anti-mouse polyvalent immunoglobulin labeled with 20-nm colloidal gold (E-Y Scientific) (2). Control sections incubated in the presence of the gold conjugate in the absence of the primary antibody were also examined to exclude the possibility that the secondary

antibody cross-reacted with developmental stages of the parasite.

Immunotherapy of suckling mice. In experiment 1, a litter of 11 4- to 5-day-old BALB/c suckling mice was used for experimental inoculations. Ten of the mice were infected per os with 10^4 oocysts of CsCl gradient-purified oocysts in a 10-μl volume. A single mouse served as an uninoculated sentinel control littermate. Beginning 24 h PI, five of the infected mice received 10 μl of supernatants containing MAb5C3 twice daily (early morning and evening) for five consecutive days. The other five infected mice received sham inoculations with control supernatants from parental myeloma cells. All mice were killed 6 days PI by CO_2 overdose, and the entire intestinal tract from the pyloric/duodenal valve to the anus of each mouse was homogenized individually in PBS in tissue grinders. The number of oocysts produced by each mouse over the 6-day interval was determined with a hemacytometer.

In experiment 2, three litters of 3- to 4-day-old BALB/c mice were used for experimental inoculations. Each litter was housed in a separate cage. All neonates in two litters were inoculated per os with 3.6×10^4 oocysts of the Auburn isolate of *C. parvum* obtained from a bovine source. One group of seven mice was treated orally at 22, 24, 26, 42, and 48 h with 20 to 25 μl of supernatant containing MAb5C3, whereas the second group of seven mice were infected but not treated. A third group of six mice served as uninoculated controls. All mice were killed with an overdose of ether at 72 h PI, and the intestines were removed, fixed in 10% neutral buffered Formalin, sectioned longitudinally, stained, and examined microscopically for developmental stages. The extent of infection was determined by using the scoring procedure devised earlier (22) and used previously by Fayer et al. (10).

All mice were housed in plastic cages with stainless-steel lids and corn cob for bedding. Mice were given commercial rodent mash and water ad libitum and kept on a 12-h light-dark cycle.

RESULTS

Antibody characterization. MAb5C3 was found to possess an IgA(κ) chain isotype. Radial immunodiffusion revealed that the antibody concentration in the supernatant was 6 μg/ml.

Electrophoresis and immunoblotting. On Western blots, MAb5C3 recognized a molecule of 14 to 16 kDa that we called GP15 (Fig. 1 and 2). Based on competition assays with various carbohydrates, it appears that *N*-acetylglucosamine and, to a lesser extent, *N*-acetylgalactosamine are important constituents of the epitope (Fig. 1). Binding was not inhibited by galactose, glucose, or mannose. In a previous study, lectin binding with concanavalin A suggested that this molecule may also possess mannose or glucose residues (30). MAb5C3 was found to recognize a similar 15-kDa epitope associated with *Cryptosporidium* sp. from guinea pigs, but binding was not observed when *C. baileyi*, *Cryptosporidium* sp. (= *C. muris* of cattle), or *C. serpentis* were probed (Fig. 2). Likewise, bradyzoites of *Besnoitia darlingi*, sporozoites of *Caryospora bigenetica*, *Eimeria nieschulzi*, and *E. papillata*, and tachyzoites of *Toxoplasma gondii* all failed to bind MAb5C3 (Fig. 2).

Fluorescent-antibody assays. Immunofluorescence assays with MAb5C3 revealed that GP15 was associated with the plasma membrane of *C. parvum* sporozoites (Fig. 3). Sporozoites within oocysts or those excysting or newly excysted

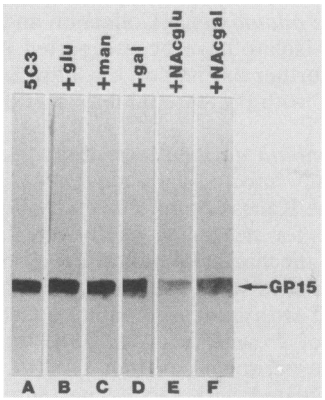

FIG. 1. Western blots of 12% SDS-PAGE of *C. parvum* oocysts and sporozoites probed with hybridoma supernatants containing MAb5C3 followed by a goat anti-mouse horseradish peroxidase-conjugated polyvalent immunoglobulin. Lanes B to F were coincubated with 200 mM competing sugars during incubation with the MAb. Lane A, Without competing sugars; lane B, with glucose (glu); lane C, with mannose (man); lane D, with galactose (gal); lane E, with *N*-acetylglucosamine (NAcglu); lane F, with *N*-acetylgalactosamine (NAcgal).

displayed an intense, evenly distributed halo over the entire sporozoite surface (Fig. 3). Once excysted, however, much of GP15 was apparently shed, since immunofluorescence was less intense on most free sporozoites. When in vitro-cultivated parasite stages were probed, diffuse fluorescence was first observed in the undifferentiated meront. As merogony proceeded, fluorescence became more pronounced and associated with the outer meront surface and then to the outer surface of budding merozoites (Fig. 4). Fluorescence was not observed in the sexual stages of the parasite.

Immunoelectron microscopy. Immunoelectron microscopy with colloidal gold labeling revealed that sporozoites, merozoites, and middle-late meronts were the only stages to bind

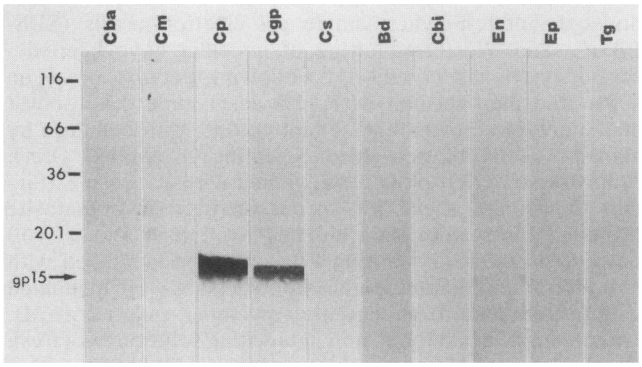

FIG. 2. Western blots of 12% SDS-PAGE of various apicomplexan parasites probed with hybridoma supernatants containing MAb5C3 followed by a goat anti-mouse horseradish peroxidase-conjugated polyvalent immunoglobulin. Molecular size markers (left) are in kilodaltons. Abbreviations: Cba, *Cryptosporidium baileyi* oocysts and sporozoites; Cm, *Cryptosporidium* sp. (*C. muris* of bovine origin) oocysts and sporozoites; Cp, *C. parvum* oocysts and sporozoites; Cgp, *Cryptosporidium* sp. from guinea pig oocysts and sporozoites; Cs, *C. serpentis* oocysts and sporozoites; Bd, *Besnotitia darlingi* bradyzoites; Cbi, *Caryospora bigenetica* oocysts and sporozoites; En, *Eimeria nieschulzi* sporozoites; Ep, *E. papillata sporozoites*; Tg, *Toxoplasma gondii* tachyzoites (RH strain).

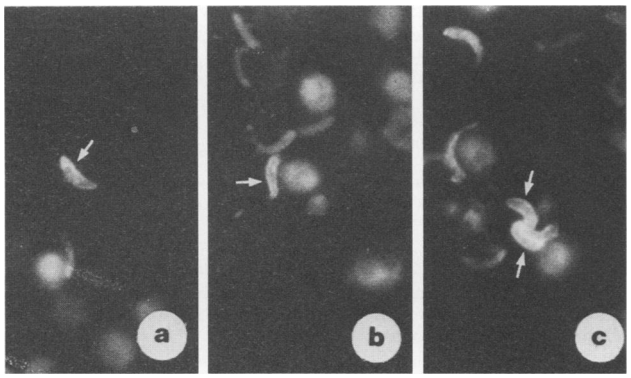

FIG. 3. Photomicrograph displaying immunofluorescence of surface of sporozoites of *C. parvum* in the presence of MAb5C3 followed by a goat anti-mouse FITC-conjugated polyvalent immunoglobulin. Magnification, ×1,200. Arrows point to excysting or newly excysted sporozoites. Note other sporozoites where epitope has been capped posteriorly.

MAb5C3. Fixation of sporozoites within oocysts was poor, but the sporozoite surfaces were observed to label intensely (not shown). Free sporozoites were also labeled, but fewer gold particles were found associated with these stages than with sporozoites within oocysts (not shown). Developmental stages associated with the ileum were of better ultrastructural quality, and gold particles were observed associated with the outer membranes of developing merozoites within meronts (Fig. 5).

Immunotherapy of suckling mice. Immunotherapy against *C. parvum* infections was tested in suckling mice. After 5 days of immunotherapy (day 6 PI), suckling mice that received twice-daily doses of MAb5C3 had a mean of 67.5% fewer oocysts than sham-treated littermates. After inoculation of 10^4 oocysts, the mean number of oocysts per animal $\pm$ 1 SD (n = 5) was 2.52 ($\pm$1.1) $\times$ 10^6 and 8.0 ($\pm$4.73) $\times$ 10^5 in the infected sham-treated and infected MAb-treated groups, respectively, versus 2.0 $\times$ 10^6 in the single uninfected control animal. Even with the high standard deviations and few animals in each group, the differences between the sham-treated and MAb-treated groups was significant at $P < 0.05$ (Wilcoxon Mann-Whitney U test). The sentinel control animal became infected, revealing that cross-transmission occurred within the cage. This is not surprising, since the dam should be able to easily transmit various numbers of oocysts among offspring by grooming. When histological sections were examined on day 3 PI, 75% fewer developmental stages were found in treated than in untreated mice (Table 1).

DISCUSSION

Western blots have shown that sera obtained from humans and animals during both active infection and the convalescence phase of cryptosporidiosis recognize a 15-kDa band from *C. parvum* oocysts and sporozoites (12, 16, 17, 19, 27, 30). Tilley et al. (27) found that this 15-kDa glycoprotein was among the most prominent antigens recognized by hyperimmune bovine colostrum (HBC), and Hill et al. (12) noted that it was strongly recognized by both serum antibodies and fecal IgA in colostrum-deprived lambs. However, the importance of GP15 as a target for immunotherapy of cryptosporidiosis has not been demonstrated experimentally until the present study.

Recently, several studies have shown that both HBC and MAbs can profoundly affect the course of *C. parvum* infections. In four cases, HBC abrogated active infections in immunosuppressed individuals, although in three cases the

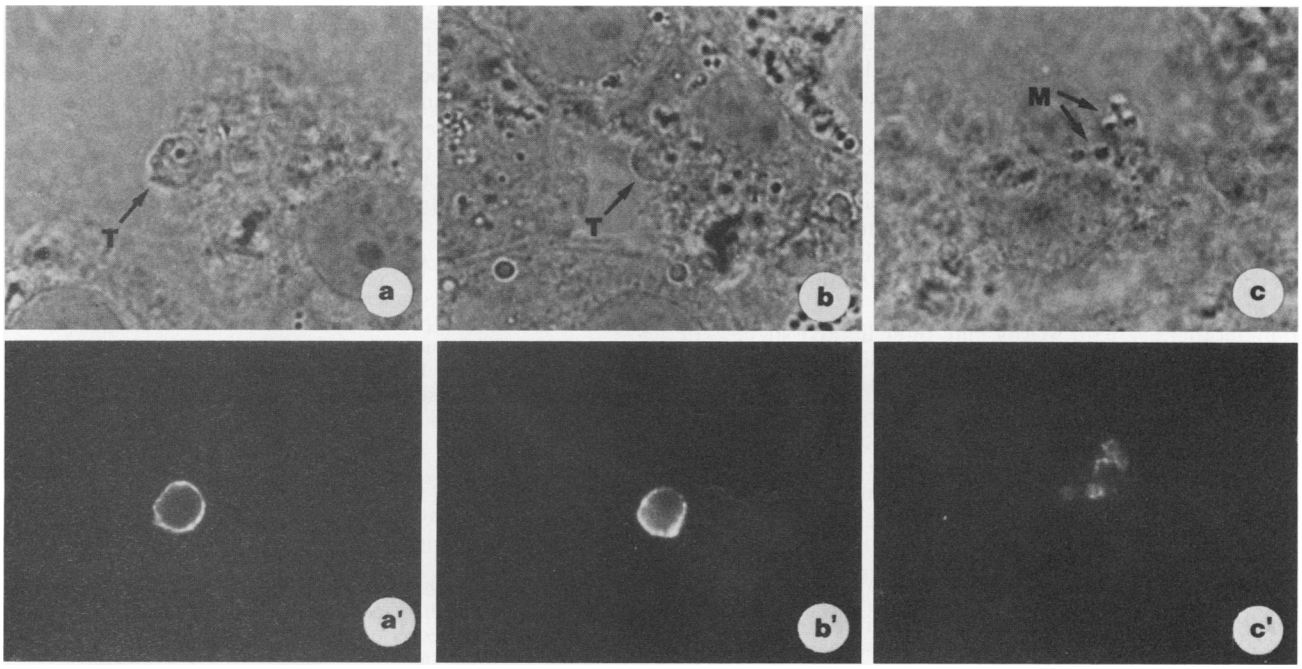

FIG. 4. Bright-field (top, a to c) and immunofluorescence (bottom, a′ to c′) photomicrographs of developmental stages of *C. parvum* cultivated in MDBK cells in vitro. (a and b) Meronts; (c) merozoites being released from meront. Magnification, ×1,500. Abbreviations: M, merozoites; T, undifferentiated meronts (trophozoites).

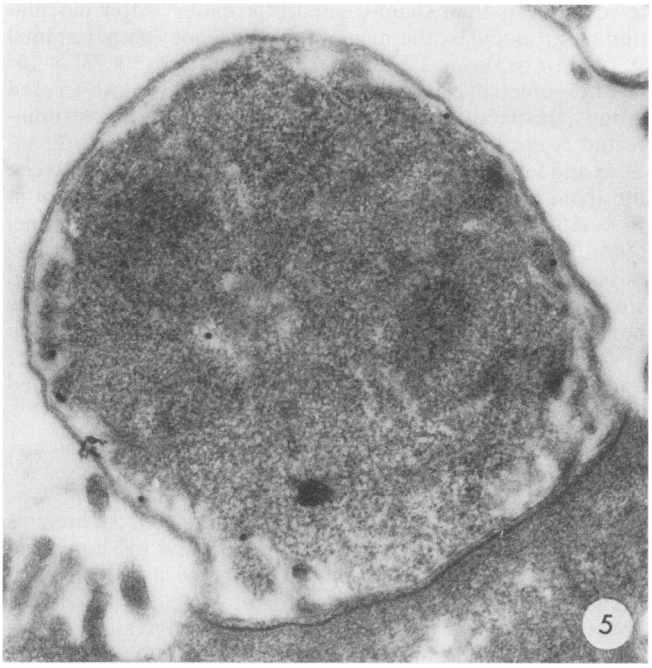

FIG. 5. Immunoelectron micrograph of developing meront of *C. parvum* with MAb5C3 and goat anti-mouse colloidal gold-labeled immunoglobulin. Gold particles are located around the periphery of the meront in association with the plasmalemma. Magnification, ×35,600.

infections started again after various lengths of time (32, 33, 38). Recrudescence was probably associated with either reingestion of oocysts or recolonization of the intestinal tract by hidden foci of organisms in the biliary or pancreatic duct. By using a mixture of three MAbs at high concentrations of 2.5 to 3.0 mg/ml, two MAbs against glycoprotein GP20 and a third that appears to be against protein P23 as well as seven or eight higher-molecular-weight molecules (P25-200 [1]), Arrowood et al. (1) effectively limited parasite development after oral administration to infected mice. These molecules have been found to be sporozoite surface molecules by MAbs, immunoelectron microscopy, biotinylation, and ^{125}I surface labeling (1, 17–19, 30). The studies reported here show that continued MAb5C3 therapy against GP15, even at doses as low as 6 μg/ml, can significantly reduce the number of developmental stages of *C. parvum* in the intestinal tract and can diminish oocyst production.

Immunotherapy with MAbs may offer limited success in treating chronic cryptosporidiosis in immunosuppressed individuals. However, the use of these antibodies as a means of short-term immunotherapy for children with persistent cryptosporidiosis may be more effective and should be considered. Reports show that *C. parvum* can often persist in children for several weeks, often leading to dehydration and electrolyte imbalance (8). Oral administration of MAb5C3 to these children might reduce the level of parasitemia until the immune system can successfully eliminate the parasite.

The finding that MAb5C3 recognizes epitopes common to both sporozoites and merozoites is not unique. Many of the MAbs generated against sporozoite surface proteins of *C. parvum* have been shown to cross-react with merozoite surface proteins, including GP20 and P23 (1, 3). These data

TABLE 1. Results of immunotherapy with MAb5C3 on numbers of developmental stages at 72 h PI in mice experimentally infected with *C. parvum*

Mouse group[a]	No. of mice	MAb5C3 dose (μl) at h PI:					Total score[b]	Mean individual score (range)
		22	24	26	42	48		
Infected, untreated	7	0	0	0	0	0	26	3.7 (3–6)
Infected, treated	7	25	20	20	20	20	6	0.9 (0–1)
Uninfected controls	6	0	0	0	0	0	0	0

[a] Each mouse, excluding controls, was inoculated with 3.6×10^4 oocysts.
[b] Sum of scores (0 to 3) for all three regions of the intestinal tract times the number of mice in each group.

help explain why passive immunotherapy may be so effective against the parasite and suggest that the parasite might actually depend upon rapid elimination by the immune system for evolutionary survival. Whereas most other species of enteric coccidia have a genetically programmed series of developmental stages in the life cycle and are incapable of recycling within the host, *C. parvum* has two stages that initiate autoinfectivity (type I merozoites and sporozoites derived from thin-walled oocysts). Without prompt elimination by the immune system, continued autoinfection would result in a prolonged, massive infection with sufficient dehydration and fluid loss to result in mortality. Considering the high seroprevalence of *C. parvum* in the world's population (35, 36), the parasite could easily eliminate itself along with most of the host population. However, we believe that the parasite is able to survive only by completing its life cycle rapidly and allowing the immune system the opportunity to eliminate specific recycling stages (sporozoites and type I merozoites). Once nonprolific differentiation into gametes has commenced, we predict that, like GP15, it would no longer be advantageous for surface antigens of sexual stages to be shared with sporozoites and merozoites. This hypothesis is further supported by our previous lectin-binding assays. Typical coccidia, such as *Eimeria nieschulzi*, have few detectable carbohydrate residues (29). Since carbohydrate epitopes associated with members of the Apicomplexa are often highly immunogenic (21), selective pressure would be against exposure of these residues on sporozoites of most coccidian species. On the other hand, *C. parvum* possesses numerous glycoproteins that can be easily detected in lectin-binding assays and which are highly immunogenic (16, 30).

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