

Anti-*Cryptosporidium parvum* Antibodies Inhibit Infectivity In Vitro and In Vivo

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***Cryptosporidium parvum* causes acute diarrhea in immunocompetent individuals and a severe life-threatening disease in immunocompromised individuals, including AIDS patients. No efficacious therapy for cryptosporidiosis has yet been reported. However, treatment of some patients with cryptosporidiosis with hyperimmune bovine colostrum has ameliorated or eliminated clinical symptoms. Consequently, it is important to identify and characterize *C. parvum* antigens which are the targets of protective antibodies to facilitate the development of more efficacious therapy. We report that hyperimmune bovine colostrum immunoglobulin inhibits *C. parvum* infectivity in a reproducible in vitro assay, and we correlate this inhibition with the protective capacity of the bovine colostrum in vivo. We have also identified the major *C. parvum* sporozoite antigens recognized on Western blots (immunoblots) by this colostrum immunoglobulin preparation. Antibodies that recognize some surface molecules of other Apicomplexan parasites are protective in vivo. Consequently, we radioiodinated membrane proteins of sporozoites and immunoprecipitated 19 molecules which are the target of immunoglobulin that is protective in vivo and in vitro.**

Cryptosporidium parvum is an Apicomplexan parasite which causes self-limited diarrhea in immunocompetent persons (36) and severe disease in immunocompromised individuals, including AIDS patients (7, 27). Disruption of either the humoral or the cellular immune response can lead to severe and prolonged cryptosporidiosis (37). Nevertheless, studies with humans and animals indicate that antibody alone can neutralize *C. parvum* infectivity. The treatment of *Cryptosporidium*-infected immunocompromised patients with hyperimmune bovine colostrum (HBC) has ameliorated or eliminated clinical symptoms (18, 32-34). Studies with experimentally infected animals indicate that immune colostrum and specific monoclonal antibodies effectively decreased *Cryptosporidium* infectivity either by passive transfer of the antibodies to infected animals or when animals were challenged with sporozoites preincubated in their presence (1, 4, 9, 20, 29). It is thus important to identify and characterize those *C. parvum* antigens which are targets of protective antibody in order to develop rational strategies for therapy of cryptosporidiosis and for vaccine development.

Surface antigens of coccidia parasites are involved in interactions with the host cell membrane during invasion of the host cell (5, 15). Apical complex organelles of sporozoites and merozoites appear to secrete their contents during host cell attachment and formation of the parasitophorous vacuole (2, 24, 28). Apical complex proteins of some coccidia have been found to be targets of protective antibody (19, 25, 26), and analogous proteins of *Cryptosporidium* spp. may elicit a similar protective response. Surface molecules of *C. parvum* sporozoites and merozoites that are recognized by antibodies which are protective in vivo (20, 31) are also promising candidate molecules for development of passive immunotherapy or vaccines. However, the identification of the relevant *C. parvum* antigens which are the target of protective antibody has been hindered by the lack of optimal experimental animal models of cryptosporidiosis and of

well-defined and reproducible in vitro assays suitable for the screening of immunotherapeutic agents (21).

We report here the correlation of inhibition of invasion and intracellular development by HBC immunoglobulin (Ig) in vitro with the protective capacity of HBC in vivo. Antibodies affinity purified from HBC Ig on Western blots (immunoblots) of *Cryptosporidium* proteins also significantly inhibited *C. parvum* infectivity in vitro, indicating that specific anti-*Cryptosporidium* colostrum antibodies neutralize parasite infectivity. Lastly, we radioiodinated sporozoite surface antigens and identified 19 surface antigens which are recognized by protective HBC Ig.

MATERIALS AND METHODS

Parasites. The *C. parvum* AUCP-1 isolate (a gift from B. Blagburn, Auburn University, Auburn, Ala.) was propagated in Holstein calves. Oocysts were isolated by resuspension of 1 volume of feces with 2 volumes of a saturated NaCl solution. All subsequent procedures were done at 4°C. After centrifugation at 1,000 × g, the supernatant was recovered and the procedure was repeated three times. Oocysts were recovered from the pooled supernatants by centrifugation, purified further in a 55-27.5-14% sucrose gradient at 1,000 × g for 20 min, and stored in phosphate-buffered saline (PBS). Prior to use, oocysts were sterilized by incubation in 15% commercial bleach and washed by repeated centrifugation and resuspension in PBS. Purified oocysts were excysted by incubation in RPMI medium (GIBCO) with the addition of 5% heat-inactivated fetal calf serum (FCS) and 0.75% sodium taurocholate (pH 7) (Sigma) for 40 to 60 min at 37°C. Sporozoites were separated from unexcysted oocysts and debris by filtration through a polycarbonate membrane (pore size, 3 μm; Millipore).

Preparation of HBC and HBC Ig. HBC (lot 40529) was prepared by ImmuCell Corp., Portland, Maine, by repeated parenteral immunizations of Holstein cows during the preparturition interval with partially excysted *C. parvum* oocysts. Immunogens were emulsified in Freund's adjuvant.

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Sham HBC (lot 41038) was prepared after immunization with a commercial herd health vaccine which was also given to cows immunized with *C. parvum* to prepare HBC (lot 40529). Colostra were collected by using standard dairy practices and frozen. A filtered (0.45- μ m-pore-size filter), lyophilized colostrum whey Ig preparation, free of low-molecular-weight solutes, was prepared from pooled colostrum from several immunized animals. Colostra were partially purified to obtain antibody products highly enriched for IgG (HBC Ig and sham HBC Ig). Colostral Ig concentrates were prepared by using large-scale production methods developed at Immucell Corp. Briefly, a pasteurized whey preparation of colostrum was prepared to eliminate the majority of caseins and fat. This preparation was then subjected to a series of ultrafiltration and microfiltration steps to remove low-molecular-weight solutes such as lactose and some whey proteins and peptides as well as particulate matter, including residual fat and caseins. The resulting concentrate was filtered, dried, and shown to be stable at room temperature. These preparations were greater than 85% protein by weight and greater than 55% IgG on a protein basis. The degree of IgG purification was at least twofold.

HBC (lot 40529) was used in the animal protection studies, and HBC Ig (50 mg of IgG per ml) of the same lot was used in the *in vitro* inhibition of development studies. Anti-*Cryptosporidium* antibody titers were determined independently several times for each colostrum preparation. HBC Ig (lot 40529) had an average anti-*Cryptosporidium* antibody titer of 1/176,000 U/ml for a 43- μ g/ μ l IgG concentration by enzyme-linked immunosorbent assay (ELISA). Sham HBC Ig (lot 41038) had an approximately 10-fold-lower average antibody titer to *Cryptosporidium* antigens by ELISA (17,000 U/ml for a 45- μ g/ μ l IgG concentration), probably as a result of natural infection of the animals in the field.

Anti-*C. parvum* antibodies eluted from Western blots. For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), 2×10^9 oocysts were lysed by five cycles of freezing-thawing in 1% Triton buffer (150 mM NaCl, 100 mM EDTA, 1% Triton X-100) in the presence of protease inhibitors (100 μ M each E64, chymotrypsin, pepstatin, and leupeptin, 1.6 mM phenylmethylsulfonyl fluoride) and boiled in sample buffer (SB). Proteins were electrophoresed in 5 to 15% gradient gels (16) and blotted onto nitrocellulose at 0.7 A for 8 h (22). Western blots were incubated with HBC Ig (lot 40529) (dilution of 1/500) in 20 ml of PBS for 3 h at 4°C and rinsed three times with PBS, and antibodies were eluted with 10 ml of glycine buffer (pH 2.6) for 3 min; then a 1/10 volume of 2 M Tris buffer (pH 8) (13) was added. Eluted antibodies were filter sterilized and concentrated to a final volume of 1 ml in a Centriprep 10 concentrator (Amicon, Lexington, Mass.).

***In vitro* inhibition assay.** We have modified an *in vitro* cell culture system (12) in order to quantify the effect of antibody on the infection of epithelial cells by *C. parvum*. MDCK cells were maintained in RPMI 1640 with the addition of 5% heat-inactivated FCS. Two-milliliter aliquots containing 2×10^5 MDCK cells per ml were seeded in eight-well tissue culture plates and allowed to attach to 20-mm² cover glasses for 24 h at 37°C in a 5% CO₂-95% air atmosphere. Cells were then rinsed in RPMI without FCS for 30 min, exposed to 2×10^6 purified oocysts resuspended in RPMI medium containing HBC Ig (lot 40529; 1,000, 500, 200, 100, and 50 μ g of IgG per ml), sham HBC Ig (lot 41038; 1,500, 250, 150, and 75 μ g of IgG per ml), eluted antibodies (50 to 100 μ g of IgG per ml), or 5% FCS (300 to 500 μ g of IgG per ml). In some experiments, controls also consisted of MDCK cells infected

with *C. parvum* oocysts resuspended in RPMI medium with the addition of glycine buffer at the same concentration used for the cultures treated with eluted antibody as described above. Cultures were incubated for 2 h at 37°C, rinsed four times with RPMI to remove extracellular sporozoites and unexcysted oocysts, and reincubated for an additional 21-h period in the presence of the respective antibody reagents as described above. Monolayers were subsequently fixed in 3.7% formaldehyde in PBS, rinsed, and stained in PBS with the addition of 1 μ M Hoescht 33258 dye (Sigma) for 1 h at 37°C (11a, 17). The number of intracellular parasites per 200 to 400 cells was quantified in three independent experiments in coded slides ($n = 3$ to 4) by fluorescence microscopy. Differences in the mean number of intracellular parasites per cell were statistically analyzed. Data were expressed as the E/C ratio (\pm standard error of the mean), where E was the mean number of intracellular parasites in the treated culture and C was the mean number of intracellular parasites in the untreated or sham HBC Ig-treated controls (6, 8).

Assessment of *in vivo* efficacy of HBC (lot 40529). Four newborn, colostrum-deprived Holstein calves were fed 100 ml of HBC plus 2 qt (ca. 1.9 liters) of commercial milk replacer at 4 h of age (treated group). Similarly, four calves were fed nonimmune colostrum (control group), and all other parameters were equal. All eight animals were challenged at 12 h of age with 5×10^6 oocysts of *C. parvum*. Animals were fed 100 ml of sham HBC or HBC every 24 h and 2 qt of milk replacer every 12 h. Clinical observations of diarrhea and dehydration were made every 12 h over a 7-day interval on all calves, and fecal samples were taken every 12 h. Fecal and dehydration scores were tabulated from days 5 to 7, the days of peak patency. Oocyst shedding was tabulated over days 5 to 9 postinfection in three of four treated animals and two of four controls. Samples from the remaining animals were not available. Oocyst shedding was measured by mixing 1 volume of fecal sample with 4 volumes of Sheather's solution and enumerating the refractive oocysts in a hemocytometer. Confirmation of the oocyst counts was performed with a commercial immunofluorescence kit, using a monoclonal antioocyst antibody (Merifluor, Meridian Diagnostics, Cincinnati, Ohio). Data were statistically analyzed by the Student *t* test.

Western blots. To identify the molecular targets of protective antibody, total *C. parvum* sporozoite and sporozoite-oocyst proteins were boiled in sample buffer, resolved in 5 to 15% gradient gels by SDS-PAGE, and Western blotted with HBC Ig. In addition, sporozoite-oocyst proteins solubilized in Triton-X 100 were immunoprecipitated with HBC Ig at dilutions of 1/1,000, 1/5,000, 1/10,000, 1/50,000, and 1/100,000. Controls were *C. parvum* proteins immunoprecipitated under the same conditions but with sham HBC Ig at dilutions of 1/1,000 to 1/10,000. Immunoprecipitates were also resolved by SDS-PAGE and Western blotted. Western blots of HBC Ig immunoprecipitates were developed with HBC Ig (dilution of 1/1,000), and sham immunoprecipitates were developed with sham HBC Ig (dilution of 1/1,000). After incubation with 10 μ Ci of ¹²⁵I-protein G for 1 h at room temperature, blots were dried and exposed for autoradiography.

Surface iodination and immunoprecipitation. Sporozoites were washed twice by resuspension in Dulbecco PBS with the addition of 1% glucose, taken to a final concentration of 10⁹ cells per ml, and radiolabeled with 300 μ Ci of ¹²⁵I-Na (Amersham, Arlington Heights, Ill.) in an Iodogen (Pierce, Rockford, Ill.)-coated glass vial as previously described (11) except that addition of 5 mM KI was omitted. Radiolabeled

sporozoites were subsequently washed three times in RPMI medium with protease inhibitors as described above. After disruption of the labeled sporozoites by five cycles of freezing-thawing, the membrane pellet and the soluble fraction containing cytoplasmic proteins were collected. An aliquot of the membrane pellet was directly boiled in SB and stored at -70°C (sporozoite membrane proteins). The remaining membrane material was divided into two radiolabeled samples. Prior to immunoprecipitation, one aliquot was extracted by boiling in SB as described above, after which 9 volumes of NETT (0.15 M NaCl, 5 mM EDTA, 0.5 M Tris, 0.5% Triton X-100 [pH 7.4]) with 1% bovine serum albumin (BSA; Sigma), 1% Triton-X 100, and protease inhibitors (SDS-solubilized membranes) was added; the other radiolabeled membrane sample was extracted directly with 3 volumes of NETT with 1% BSA, 1% Triton X-100, and protease inhibitors (Triton X-100-solubilized membranes). Both samples were precleared by addition of 1 μl of sham HBC Ig followed by overnight incubation at 4°C . Subsequently, 200 μl of protein G-Sepharose 4B beads was added, and the samples were rocked for 1 h at room temperature. After centrifugation at $10,000 \times g$, HBC Ig affinity bound to protein G-Sepharose 4B beads (300 μl) was added to the supernatants, and the samples were rocked for an additional 2 h at 37°C . Immunoprecipitates were washed sequentially with NETT buffer alone and NETT containing 1% BSA (Sigma) or 500 mM NaCl, then boiled in SB, and stored at -70°C . Proteins were separated in 5 to 15% gradient gels by SDS-PAGE and processed for autoradiography using X-Omat film (Kodak). Iodination controls consisted of trichloroacetic acid precipitates of the soluble fraction containing sporozoite cytoplasmic proteins which were also processed as described above.

RESULTS

An *in vitro* assay was used to determine inhibition of *Cryptosporidium* invasion and/or intracellular development as a function of HBC Ig titer. HBC Ig, at concentrations ranging from 100 to 1,000 μg of IgG per ml, resulted in a significant (up to 61%) reduction in the mean number of intracellular parasites per host cell relative to FCS controls (Fig. 1) ($P < 0.01$), while no inhibition was observed at lower HBC Ig (≤ 50 μg of IgG per ml) concentrations. We also evaluated a second control, sham HBC Ig, and found that it did not significantly inhibit *C. parvum* infectivity relative to 5% FCS controls, although this reagent does contain some anti-*Cryptosporidium* activity by ELISA (Fig. 2). In addition, HBC Ig (100 to 1,000 μg of IgG per ml) significantly (by 45 to 55%) inhibited *Cryptosporidium* invasion and/or development compared with sham HBC Ig controls. Specific anti-*C. parvum* antibodies were affinity purified from Western blots (~ 50 to 100 μg of IgG per ml). This eluted antibody also inhibited *Cryptosporidium* invasion and/or development relative to sham HBC Ig controls and controls with the addition of glycine buffer or FCS ($P < 0.01$) (Fig. 3).

The previous *in vitro* results were confirmed in a newborn calf model of acute cryptosporidiosis in which HBC (lot 40529) was shown to be protective. Protective efficacy of the immune colostrum preparation was demonstrated in statistically significant differences between treated and control animals in cumulative fecal scores ($P < 0.01$) and dehydration scores ($P < 0.025$ by one-tailed *t* test). No dehydration occurred in the treated group, and oocyst output was significantly reduced to below the limit of detection in the treated group ($< 10^3$ oocysts per total fecal output) compared with

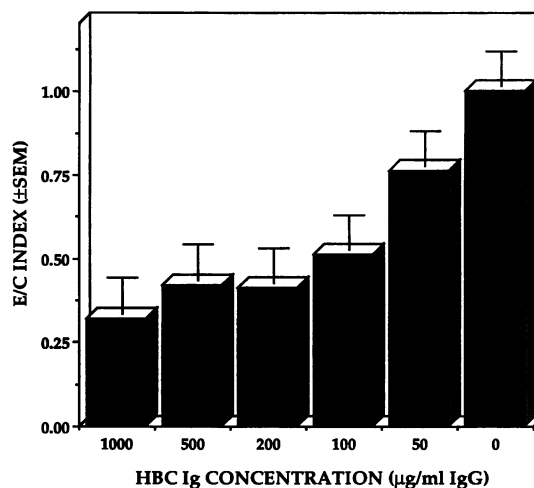


FIG. 1. Inhibition of *Cryptosporidium* infection of epithelial cells by HBC Ig (lot 40529). Antibody concentrations ranging from 100 to 1,000 μg of IgG per ml resulted in a significant reduction ($P < 0.01$) in the mean number of intracellular parasites per MDCK cell of up to 61% relative to FCS and of up to 55% relative to sham HBC Ig controls, while no inhibition was observed at lower HBC Ig concentrations (≤ 50 μg of IgG per ml).

the controls (geometric mean = 5.62×10^8 oocyst output). These results indicate that the immune colostrum treatment was effective in reducing initial colonization as well as suppressing intestinal proliferation of those organisms which were not initially neutralized.

When total oocyst-sporozoite proteins were immunoprecipitated with HBC Ig at different concentrations and blotted, two sporozoite molecules of >900 and ~ 250 kDa were the major antigenic targets identified by protective colostrum (Fig. 4, lanes 1 to 4) but not by sham HBC Ig (lanes 5 to 7) at all antibody concentrations assayed. Most of the *C. parvum* antigenic proteins recognized by HBC Ig are expressed by sporozoites, as evidenced by the comparison of total sporozoite and oocyst-sporozoite proteins recognized by HBC Ig in Western blots (data not shown).

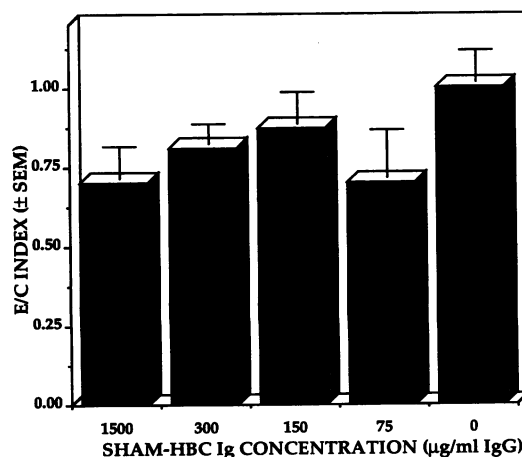


FIG. 2. Effect of sham HBC Ig on *C. parvum* infection of MDCK cells. No significant inhibition was observed for sham HBC Ig at concentrations ranging from 1,500 to 75 μg of IgG per ml with respect to FCS controls ($P < 0.01$).

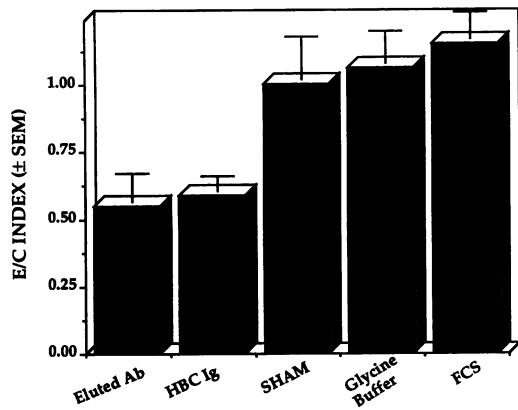


FIG. 3. Specific anti-*Cryptosporidium* antibody assay. HBC Ig antibodies (50 to 100 μ g of IgG per ml) were eluted from *C. parvum* Western blots and used in the MDCK in vitro assay. In this case, experimental data were normalized to those for sham HBC IgG controls (75 μ g of IgG per ml). Significant inhibition of *Cryptosporidium* infectivity was observed for cultures treated with anti-*Cryptosporidium* antibodies eluted from Western blots (Eluted Ab) and HBC Ig (100 μ g of IgG per ml) (HBC Ig) with respect to sham HBC Ig (100 μ g of IgG per ml) (SHAM), glycine buffer, and FCS controls ($P < 0.01$).

Cryptosporidium sporozoites were also radioiodinated to identify antigens localized to their surface. Twenty-two surface-iodinatable sporozoite proteins were resolved by SDS-PAGE (Fig. 5, lane 1). Protective anti-*Cryptosporidium* antibodies (lot 40529) immunoprecipitated most of these surface-labeled sporozoite proteins. The number of radiolabeled immunoprecipitated proteins resolved by gel electrophoresis differed for membranes solubilized with SDS or with Triton X-100. Nineteen labeled sporozoite surface proteins extracted with SDS were specifically immunoprecipitated by HBC Ig (Fig. 5, lane 2), including >900- and

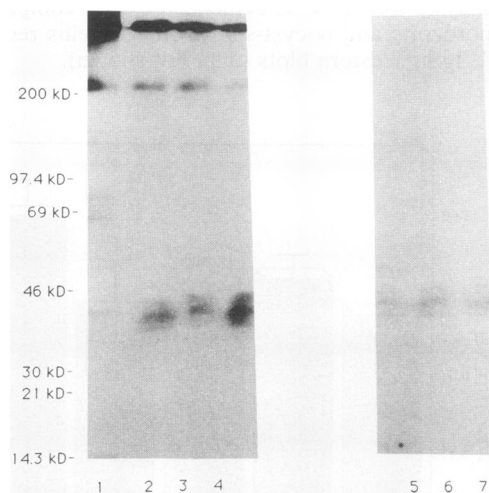


FIG. 4. Western blot of *C. parvum* proteins developed with HBC Ig or sham HBC Ig. Oocyst-sporozoite proteins were either blotted (lane 1) or immunoprecipitated with HBC Ig at dilutions of 1/1,000 (lane 2), 1/5,000 (lane 3), and 1/10,000 (lane 4) and with sham HBC Ig at the same dilutions as specified above (lanes 5 to 7). After electrophoresis, Western blots were developed with HBC Ig (lanes 1 to 4) or sham HBC Ig (lanes 5 to 7) followed by 125 I-protein G and then autoradiographed.

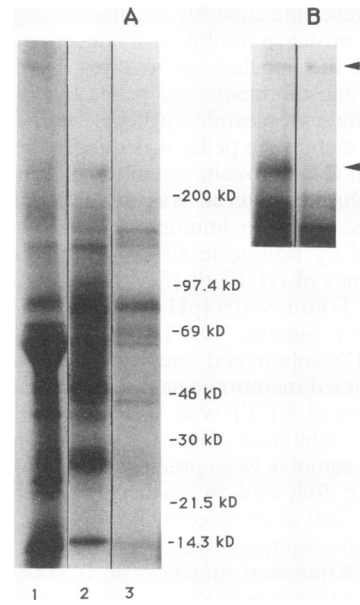


FIG. 5. (A) Surface proteins of *C. parvum* sporozoites recognized by HBC Ig. Radioiodinated sporozoites were disrupted, and an aliquot of the membrane proteins was directly resuspended in SB and electrophoresed (lane 1). Alternatively, radiolabeled sporozoite membranes were SDS solubilized (lane 2) or Triton X-100 solubilized (lane 3) prior to immunoprecipitation with affinity-bound HBC Ig. Proteins were resolved by SDS-PAGE and autoradiographed for 8 h. (B) Detail of autoradiogram of lanes 2 and 3 exposed for 24 h. The >900- and ~250-kDa molecules are indicated by arrows.

~250-kDa molecules (Fig. 5B). Only 13 radioiodinated 1% Triton X-100-soluble proteins were immunoprecipitated by protective HBC Ig (Fig. 5, lane 3), including the >900-kDa species (Fig. 5B). No radioiodinated molecules were observed in trichloroacetic acid-precipitated controls of the soluble cytoplasmic fraction (data not shown), indicating that only membrane proteins were iodinated by this methodology.

DISCUSSION

Immunotherapy of cryptosporidiosis is a promising treatment, as intraluminal gastric administration of HBC to immunocompromised patients with cryptosporidiosis effectively reduced symptoms (18, 32-34). Fayer et al. (9) demonstrated that Ig fractions within the colostrum were responsible for sporozoite neutralization. However, variability in the effectiveness of the treatment of humans and animals with HBC, which might partially reflect differences in the titers of protective antibody, has been reported (14, 18, 32). Consequently, there is a need to identify and characterize the antigenic targets of protective humoral immunity in order to develop rational anti-*Cryptosporidium* immunotherapy for immunocompromised patients. With this purpose, we have correlated the protective capacity of an HBC preparation in vivo with significant inhibition of infection in a reproducible epithelial cell-*Cryptosporidium* assay. Inhibition of *C. parvum* intracellular development in vitro occurred as a function of anti-*Cryptosporidium* antibody titer, as evidenced by its correlation with Ig concentration in protective colostrum and by the lack of biological activity of sham colostrum. Moreover, HBC Ig also significantly inhibited *C. parvum* infectivity in the Caco-2 cell line (5a). This in vitro assay will

allow the preliminary testing of potential anti-*Cryptosporidium* therapeutic reagents prior to their evaluation in experimental animal models. Confirmation that the protective capacity of HBC in vitro is in fact mediated by specific anti-*Cryptosporidium* antibodies and not by other antibodies or components comes from the inhibitory effect of antibodies eluted from Western blots of sporozoite-oocyst proteins. A previous report evaluated the efficacy of a soluble fraction of unknown Ig concentration of an HBC reagent for its ability to inhibit *Cryptosporidium* infection of HT29.74 cells (10), and similar in vitro inhibitory rates were observed, although these results were not correlated with the protective capacity of the reagent in vivo. In our study, the in vitro results were confirmed in a newborn calf model of acute cryptosporidiosis in which HBC (lot 40529) was shown to be protective. Oocyst output was reduced to below the limit of detection, and no dehydration occurred in the HBC-treated animals. Moreover, this HBC Ig preparation was also protective in a therapeutic neonatal mouse model of cryptosporidiosis (5a), confirming that immune colostrum treatment effectively reduces *Cryptosporidium* infectivity and/or proliferation.

Cryptosporidium antigens recognized by specific antibodies present in this HBC preparation were identified by Western blotting. The majority of the antigenic molecules were expressed by the sporozoite and were specifically immunoprecipitated by HBC Ig and not by sham colostrum. Similar complex patterns of sporozoite and oocyst proteins recognized by bovine colostrum antibodies have been reported for another *C. parvum* isolate (29). In our Western blots with the AUCP-1 isolate, two bands of >900 and approximately 250 kDa were the major targets of protective HBC antibody. To identify *Cryptosporidium* antigens localized to the surface of the sporozoite, parasites were iodinated by a protocol that allows the specific labeling of surface components (11). In agreement with a previous report which identified surface-iodinatable proteins of the *Cryptosporidium* KSU-1 isolate (30), we identified approximately 22 sporozoite surface proteins by electrophoresis. In contrast with a previous report on *Eimeria* surface proteins analyzed by electrophoresis (35), similar patterns of surface iodinated proteins were obtained when *Cryptosporidium* oocysts were excysted in complete RPMI medium in the presence or absence of taurocholate (7a). These differences may be related to variations in the excystation methods used or, alternatively, reflect differences in the solubility of sporozoite surface proteins of these Apicomplexan parasites. Two radioiodinated molecules of the same M_r as described above were immunoprecipitated by affinity-bound HBC Ig from SDS-solubilized sporozoite membranes. One of these surface proteins was identified as GP900 (7a), a Triton X-100-soluble glycoprotein that is highly antigenic and abundant in sporozoites (22, 23). GP900 is localized to the anterior end of the sporozoite and also appears to be expressed by merozoites (22). As this molecule is present in both parasite stages which are free in the lumen of the gut and available for antibody neutralization, we are currently evaluating the protective capacity of specific anti-GP900 antibodies. The development of the quantitative in vitro system of inhibition of infection and the feasibility of affinity purifying antibodies directed to specific antigens of the sporozoite should allow us to identify the relevant surface antigens of cryptosporidia which are the target of protective antibodies, to characterize these antigens, and eventually to develop specific immunotherapeutic reagents for treatment of this medically important parasitic disease.

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