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Immunotherapeutic Efficacy of Bovine Colostral Immunoglobulins from a Hyperimmunized Cow against Cryptosporidiosis in Neonatal Mice

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Infection with *Cryptosporidium parvum*, a ubiquitous protozoan parasite of virtually all mammals, can cause mild to severe diarrhea in immunocompetent hosts and life-threatening diarrhea in immunocompromised hosts. Passive immunotherapy of experimentally infected animals and naturally infected humans with hyperimmune bovine colostrum has been reported to be efficacious, whereas chemotherapy has not. In this study, the efficacy of specific immunoglobulin isotypes purified from bovine colostrum from a cow hyperimmunized with *Cryptosporidium parvum* was assessed in neonatal BALB/c mice. Mice were orally infected with oocysts and treated with whole whey immmunoglobulin G1 (IgG1), IgG2, IgA, or IgM at six intervals from 22 to 66 h postinfection. In histologic sections of intestine examined at 72 h postinfection, the reduction in number of intestinal stages in treated mice versus untreated controls was very highly significant ($P < 0.0001$). The greatest reduction in parasite number was found in mice treated with IgG1, IgA, or whey.

Cryptosporidium parvum is a ubiquitous, zoonotic, coccidian parasite of mammals throughout the world (3). Spread by the fecal-oral route, it primarily parasitizes intestinal epithelium and to a lesser extent extraintestinal epithelia and causes diarrhea which may be mild to severe in immunocompetent animals and humans but can be life threatening to immunocompromised individuals (3). Of nearly 100 chemotherapeutic regimens tested, none have been found effective (3). Epidemiologic studies on diarrhea from *C. parvum* in Costa Rica, Ecuador, Guatemala, Haiti, and Liberia indicated less cryptosporidiosis in breast-fed infants than in non-breast-fed infants in the same populations (2, 6–9, 12). The concept that lactogenic factors could prevent or cure cryptosporidiosis was challenged when milk or colostrum from mice that had been naturally infected with *C. parvum* and then immunologically boosted by oocyst challenge, and therefore was assumed to be immunized, failed to provide passive prophylaxis for the suckling pups against experimental cryptosporidiosis (1, 11) and when bovine colostrum containing anti-*Cryptosporidium* antibody (albeit at a very low level) failed to provide therapeutic relief for human cryptosporidiosis (15). Reflecting on these results, some reasoned that the lower incidence of cryptosporidiosis in breast-fed children might be explained as resulting from their lack of exposure to contaminated water or other environmental factors rather than from any anticryptosporidial activity by lactogenic factors. Other studies revealed that colostrum with very high titers of anticryptosporidial antibody produced by cows hyperimmunized by multiple injections of *C. parvum* into the mammary gland provided efficacious passive prophylaxis and therapy against cryptosporidiosis in both animals and humans (4, 5, 18–20). However, because whole colostrum or whey was used in these studies, it was not known which constituents—cytokines, immunoglobulins, or other factors—were responsible for the efficacy. This study was undertaken to elucidate factors in hyperimmune colostrum that might be efficacious against *C. parvum* in experimentally infected mice. By using whole whey and affinity-purified immunoglobulin G1 (IgG1), IgG2, IgM, and IgA in separate test groups, we demonstrated the activity of sporozoite- or oocyst-induced lactogenic immunoglobulins against endogenous stages and suggest that other factors possibly present in whole colostrum may not be essential for the anticryptosporidial activity.

MATERIALS AND METHODS

Two replicate studies were conducted. The design for each replicate was based on treating each litter of neonatal mice as a separate treatment group so that six dams with litters were each treated as one of groups 1 through 6. In replicates 1 and 2, four or five pups in each group received an oral inoculation of 250,000 *C. parvum* oocysts (AUCP1 isolate) in 25 or 10 μl of Hanks balanced salt solution (HBSS), respectively. At 22 and 24 h postinfection (p.i.), all pups received 25-μl amounts of the treatments orally. Group 1 received whole whey, group 2 received IgG1, group 3 received IgG2, group 4 received IgM, group 5 received IgA, and group 6 received HBSS. At 26, 42, 48, and 66 h p.i., 25-μl amounts of the same solutions were injected intraintestinally by rectal insertion of 1 cm of polyethylene tubing attached to a 27-gauge needle fitted to a 25-μl syringe (Hamilton Co., Reno, Nev.). The decision to administer 50 μl of treatment orally and 100 μl intraintestinally was made after observing that not all oral doses were immediately swallowed, that unequal treatment could result, and that attempts at gastric intubation of such tiny pups might result in physical harm. At 72 h, 49 mouse pups in the two replicates were euthanized with ether. The intestine was immediately removed, cut in 1.0- to 1.5-cm lengths, arranged sequentially on spongettes in tissue capsules, and fixed in 10% neutral buffered Formalin. Hematoxylin-eosin-stained

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longitudinal sections were examined microscopically without knowledge of treatment group. Scores were assigned on the basis of infectivity as previously described (14). Basically, scores of 0, 1, 2, and 3 represented absence of infection (no parasites), 33% or less of the mucosa parasitized, 34 to 66% of the mucosa parasitized, and 67% or more of the mucosa parasitized, respectively. Cumulative scores for the ileum, cecum, and colon were determined for each pup so that a pup, for example, with no parasites in the ileum, 33% of the cecal mucosa parasitized, and over 67% of the upper colonic mucosa parasitized would score 0, 1, and 3, for a cumulative score of 4. Mean scores and standard errors are shown graphically in Fig. 1. Data were statistically analyzed by using regression analysis provided by the general linear models procedure of the statistical analysis system, using treatment as the main effect. Specific comparisons between treatments and controls were made by using contrast statements. Statistically significant events were present when the probability was less than 0.05.

Hyperimmune colostrum. A pregnant, multiparous Holstein-Friesian cow was obtained from the Beltsville Agricultural Research Center Dairy Unit 12 weeks before parturition (WBP) and housed in a cinderblock building (20 by 20 ft [ca. 6.1 by 6.1 m]) with free access to a fenced yard. At 10 WBP, she received an intramuscular injection in the left hind leg with 20×10^6 *C. parvum* oocysts. Oocysts were less than 1 month old when used. They were obtained from the feces of dairy calves, were cleaned by sucrose density flotation techniques, and were sterilized with sodium hypochlorite as previously described (5). They were frozen and thawed three times in 5 ml of HBSS and then emulsified with 5 ml of Freund complete adjuvant. At 8, 6, and 4 WBP, she received intramammary infusions through the teat canals of all four quarters of the udder; the infusions consisted of 1 ml of frozen-thawed (three times) oocysts in HBSS emulsified with an equal volume of Freund incomplete adjuvant. At 8, 6, and 4 WBP, each of the four infusions contained 2×10^7, 5×10^6, and 5×10^6 oocysts, respectively. Colostrum obtained from the first milking after parturition was immediately frozen at −20°C.

Whey and immunoglobulin preparation. Whey was prepared by thawing the frozen colostrum and then centrifuging 50-μl samples at 45,000 × *g* for 1 h at 4°C. Butter fat was removed and whey was aspirated, leaving the pelleted cellular debris and casein. The whey was diluted 1:1 with 0.01 M phosphate-buffered saline (0.85% saline, pH 7.4) and centrifuged, residual fat was removed, and the whey was aspirated from the pellet. The whey was dialyzed against distilled water overnight to precipitate euglobulins. The euglobulin precipitate was removed by centrifugation at 43,000 × *g* for 30 min at 4°C and used to prepare purified IgM; the supernatant was used to prepare IgG1, IgG2, and IgA.

Euglobulin precipitate was dissolved in starting buffer (0.01 M phosphate, 0.25 M NaCl, pH 7.0) and absorbed with monoclonal antibody (MAb) (16), specific for IgM, bound to Sepharose 4B. Sepharose was washed, first with starting buffer and then with 0.01 M phosphate buffer (pH 2.6), and immediately adjusted to pH 7.0 with 1 M NaOH. Eluted IgM was tested for purity in an enzyme-linked immunosorbent solid-phase assay, using MAb to bovine IgG1, IgG2, IgA, and IgM. Contaminating isotypes were removed by absorption with the respective isotype-specific MAb bound to Sepharose 4B.

Supernatant from the H_2O dialysis was diluted 1:5 in starting buffer before absorption with the respective bovine isotype-specific MAb (IgG1, IgG2, and IgA) bound to Sepharose. Procedures for washing, elution, and purity testing were the same as described for IgM.

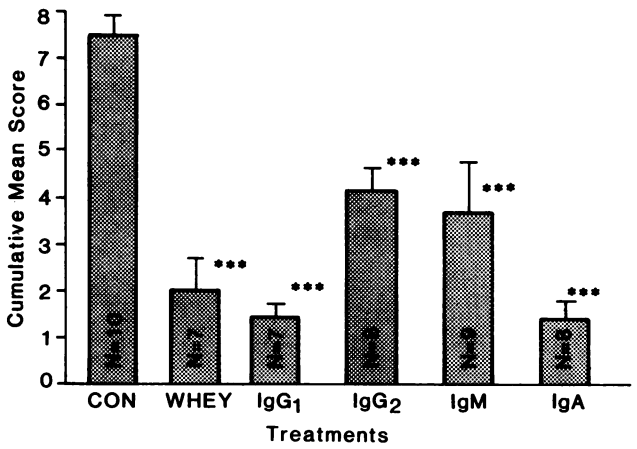

FIG. 1. Graphic representation of statistical analysis. Group means and standard errors are presented for each treatment group. *** indicates statistically significant difference from the control group (CON) ($P < 0.0001$).

Isotypes were adjusted to their respective starting concentration in colostral whey, as determined from enzyme-linked immunosorbent assay values.

Mice. Each BALB/c dam with her 2- to 3-day-old litter (Harlan, Sprague Dawley, Cincinnati, Ohio) was housed in a large rat cage fitted with a filter top and inserted into a cage rack that provided filtered air to each cage. Pelleted feed and water were available at all times. Although replicate studies began with four or five pups per litter, in some groups only three or four pups survived until the experiment terminated. In these cases, it appeared that the dam either refused to nurse the pup or killed it. Pups were 3 to 4 (replicate 1) or 6 to 7 (replicate 2) days old when inoculated with oocysts. Pups were removed from their dams 30 min before oral treatments and immediately before intraintestinal treatments. They were returned immediately after treatment. All procedures were conducted in a laminar-flow hood.

RESULTS AND DISCUSSION

Group mean cumulative scores (replicates 1 and 2 combined) for infected epithelial cells in the ileum, cecum, and colon of the neonatal BALB/c mice 72 h after infection with *C. parvum* are shown in Fig. 1. Mice in the infected, HBSS-treated control group were heavily infected at all three locations, with a high mean score of 7.5 ($n = 10$; Fig. 1 and 2). In comparison, every group treated with whey or an individual immunoglobulin constituent had significantly fewer stages of *C. parvum* in the histologic specimens than did the control group ($P > 0.0001$). Group mean cumulative scores (replicates 1 and 2 combined) for treatments with whey, IgG1, IgG2, IgM, and IgA were 2.00 ($n = 7$), 1.43 ($n = 7$), 4.13 ($n = 8$), 3.67 ($n = 9$), and 1.38 ($n = 8$), respectively. Those treated with whey, IgG1, or IgA (Fig. 3) had even fewer stages present than those treated with IgG2 or IgM.

The high mean cumulative scores for infected untreated control mice confirm the infectivity of the *C. parvum* oocyst inoculum and provide an index of the relative extent of infection (Fig. 1 and 2). The scores for all groups that received the whole whey or any of its constituent immuno-

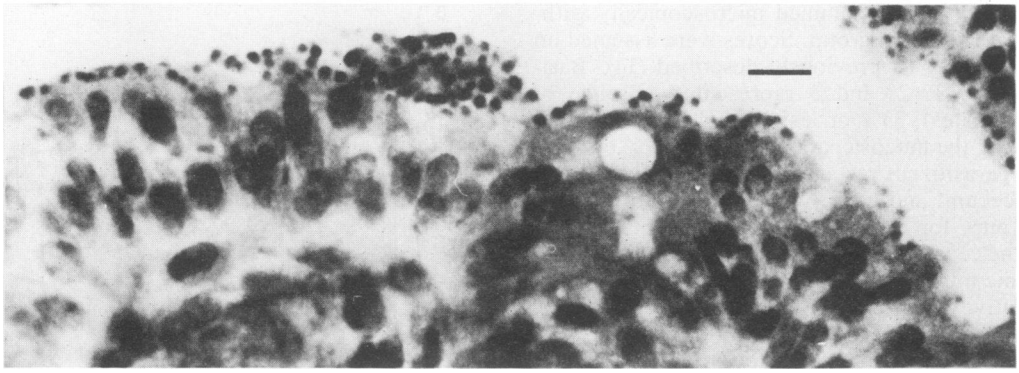

FIG. 2. Photomicrograph of ileum of untreated control mouse 72 h p.i. Spherical bodies at the surface of cecal epithelial cells are developmental stages of *C. parvum*. Cumulative infection score of 9; bar, 25 μm.

globulins were significantly lower than scores for controls ($P < 0.0001$), indicating that administration of any immunoglobulin alone or in combination in whey reduced the extent of or totally eliminated the parasite (Fig. 1 and 3). Until now, it was known that colostrum from cows hyperimmunized by the protocol used in this study provided prophylactic protection to calves (4), neutralized sporozoites and oocysts given to mice (5, 13), and provided therapy to chronically infected humans (18–20). However, because only whole colostrum or whole whey had been used in these studies, it was not known whether immunoglobulins, cytokines, or other factors were responsible for the antiparasitic activity. This study clearly indicates that purified immunoglobulins alone possess such activity. Because it was demonstrated that colostral whey from a cow immunized with oocysts of *C. parvum* and given undiluted, diluted 1:20, or diluted 1:50 to neonatal BALB/c mice before, during, and after oral inoculation with oocysts of *C. parvum* significantly reduced the number of parasites that developed in those mice compared with mice treated similarly with colostrum from a nonimmunized cow or with HBSS (5), controls using whey from a nonimmunized cow were omitted from this study. Virtually all cows have been exposed to *C. parvum* during their lifetimes and demonstrate some positive antibody in colostral whey. The objective of this study was to determine whether the immunoglobulins in whey from a known hyperimmunized cow could affect the development of *C. parvum* stages. The study was not designed and did not resolve the issue of whether active factors other than the immunoglobulins might be present in the whey.

Because the immunization protocol involved only frozen-thawed oocysts that contained sporozoites, it might be expected that antibodies produced in response to this exposure would be targeted against stage-specific antigens associated with the sporozoite/oocyst stage. Because the period of administration of whey and immunoglobulins extended from 22 to 66 h p.i., a period in the life cycle of the parasite when neither sporozoites nor oocysts are known to be present, it appears likely that one or more of the endogenous stages (meronts, merozoites, or gamonts) must have antigens recognized by the sporozoite- or oocyst-induced antibodies.

Until endogenous stages of *C. parvum* become available either through the advent of improved in vitro cultivation techniques or via isolation of stages from gut epithelium as has been accomplished with some poultry coccidia, the stage-specific antigens recognized by the immunoglobulins in this study can be identified only by indirect methods. Western immunoblots using the sporozoite stage and whole whey from the same cow used in this study (no. 8801) were probed with rabbit polyclonal serum to specific bovine immunoglobulin isotypes to identify which sporozoite antigens were recognized by which immunoglobulins (17). Of the seven colostrum samples tested from hyperimmunized cows (17), the colostrum used in this study was one that reacted only moderately with *C. parvum* protein bands, i.e., less intensely than some samples and more intensely than others. Although all four colostral immunoglobulin isotypes from cow 8801 recognized many bands, overall, IgA and IgG1 reacted more intensely and with more bands than did IgG2 and IgM (17), providing a rationale for the finding in this study of a greater reduction in parasite number in mice treated with IgA and IgG1 than in those treated with IgG2 and IgM. For cow 8801, IgA prominently recognized bands

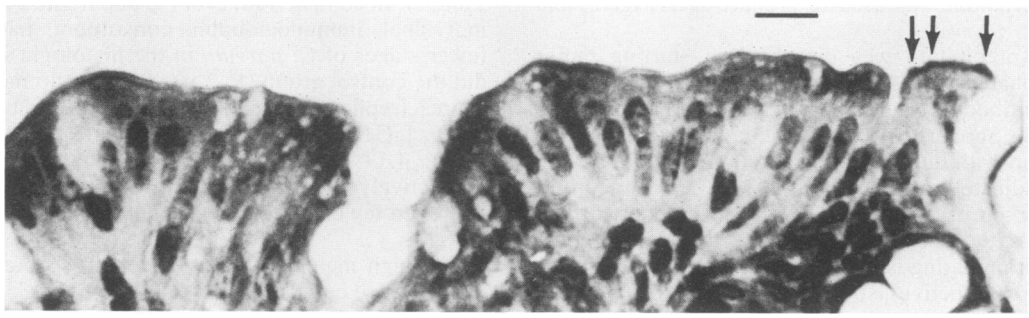

FIG. 3. Photomicrograph of ileum of IgA-treated mouse 72 h p.i. Only three parasites are barely evident at the surface of cecal epithelial cells. Cumulative infection score of 1; bar, 25 μm.

at 9 to 10, 38 to 40, 41 to 43, 49, 68 to 72, 88, 100, and 147 kilodaltons (kDa); IgG1 prominently recognized bands at 14.5 to 16.5, 38 to 40, 45 to 46, 49, 56, 68 to 72, 88, 100, 127, and 147 kDa; IgG2 recognized bands at 14.5 to 16.5, 68 to 72, 88, 127, and 147 kDa; and IgM recognized bands at 88, 100, and 147 kDa (17).

Similar immunotherapeutic efficacy of antibody was demonstrated by a different approach. An MAb that reacted with a 20-kDa sporozoite surface determinant (10) as well as purified MAb mixtures and ascites fluid mixtures that reacted with multiple antigens reduced the parasite loads in experimentally infected mice receiving daily oral treatments (1). MAb that recognize other proteins remain to be tested.

Radiolabeling of surface antigens before Western blotting or electron microscopy of developmental stages exposed in situ to whey (or immunoglobulins) followed by immunogold labeling to identify the binding site(s) could identify surface antigens recognized by colostral antibodies, aiding in the selection of proteins for future immunologic testing.

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