Protection of calves against cryptosporidiosis with immune bovine colostrum induced by a *Cryptosporidium parvum* recombinant protein

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Abstract

The purpose of the study was to determine if immunization with a recombinant protein (rC7) of *Cryptosporidium parvum* would induce immune bovine colostrum that protected calves against cryptosporidiosis following oral challenge with *C. parvum* oocysts. Late gestation Holstein cows with low titers of antibody to the p23 antigen of *C. parvum* were immunized three times with 300 μg affinity purified rC7 *C. parvum* recombinant protein (immune cows), or left nonimmunized (control cows). Colostrum was obtained from each cow in both groups and partitioned into identical aliquots of pooled immune colostrum or pooled control colostrum. Twelve calves obtained at birth received either immune or control colostrum within the first 2 h, and again at 12 and 24 h of age. Each calf was challenged orally with 10⁷ *C. parvum* oocysts at 12 h of age and monitored for signs of cryptosporidiosis. All six calves administered pooled control colostrum developed severe diarrhea (mean total fecal volume = 8447 ± 5600 ml) and shed an average of 1.87 ± 1.66 10¹² *C. parvum* oocysts. None of the six calves administered pooled immune colostrum developed diarrhea (mean total fecal volume = 740 ± 750 ml, p < 0.05), and shed significantly fewer oocysts (3.05 ± 2.26 x 10⁹, p < 0.05). The absence of diarrhea and 2.79 log₁₀ (99.8%) reduction in oocyst excretion indicates that immune bovine colostrum induced by immunization with *C. parvum* recombinant protein rC7 provided substantial protection against cryptosporidiosis in neonatal calves. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Cryptosporidiosis is a widespread diarrheal disease of humans, young calves and many other mammals caused by infection of intestinal epithelial cells with the protozoal agent *Cryptosporidium parvum* [1-4]. The disease occurs in humans of all ages. *C. parvum* is one of the causes of diarrhea in travelers, and is frequently transmitted among young children in day care centers. Disease in these groups, while unpleasant and sometimes severe, is usually self-limiting [5,6]. In contrast, cryptosporidiosis in immunocompromized patients is persistent, severe and often life-threatening [5,7,8]. Management of infection in immunodeficient hosts is complicated by absence of chemotherapeutic agents with consistent efficacy against the organism [9]. The disease in cattle and most other mammals is limited to neonates [2,10,11]. Older calves are resistant to cryptosporidiosis, but the mechanisms for age-related susceptibility to infection are unknown [10].

Infection of susceptible hosts follows ingestion of *C. parvum* oocysts which excyst within the intestinal tract and release sporozoites capable of binding to and penetrating intestinal epithelial cells [2]. The subsequent asexual portion of the life cycle yields mero-
merozoites that are released into the intestinal lumen and have the ability to infect additional epithelial cells [2, 12]. Completion of the sexual phase of the life cycle results in production and release of two types of oocysts into the intestinal lumen. Thin walled oocysts may excyst within the host and release sporozoites that initiate a new cycle of infection. Thick walled oocysts are excreted to the environment and serve as a source of infectious organisms for new hosts.

We reasoned that colostrum obtained from cows immunized with appropriate C. parvum antigens should neutralize a sufficient number of sporozoites within the intestinal lumen of calves to protect them against clinical manifestations of C. parvum infection. If so, a recombinant C. parvum protein expressing neutralization-sensitive epitopes may be effective in stimulating production of protective colostrum. We selected rC7, a recombinant protein containing the amino acid sequence of the major part of p23, an immunodominant surface protein found on C. parvum sporozoites and merozoites [13–16]. This recombinant protein contains at least two neutralization-sensitive epitopes, one of which is composed of the linear amino acid sequence QDKPAD [13]. The purpose of this study was to determine if immunization with C. parvum rC7 would induce immune bovine colostrum that protected calves against cryptosporidiosis following oral challenge with 10⁷ C. parvum oocysts.

2. Materials and methods

2.1. Parasites

The Iowa isolate of C. parvum, originally obtained from H. Moon (NADC, Ames, IA), was used for calf challenge experiments. Oocysts isolated from feces of infected calves were purchased from Pleasant Hill Farm (Troy, ID), and used within 12 weeks of purchase. Oocyst excystation was tested at the time that each calf was challenged, and always exceeded 90% [17].

2.2. Recombinant protein rC7

The C. parvum recombinant protein rC7 was obtained from a cDNA library as previously described [13]. The recombinant protein contains 187 amino acids, the C terminal 101 of which are the same as the C terminus of p23, an immunodominant surface protein expressed by C. parvum sporozoites and merozoites [13, 14].

Recombinant C7 protein used for immunization of cows was produced using a glutathione S-transferase prokaryotic protein expression system (Amersham Pharmacia Biotech, Arlington Heights, IL) [18]. C7 DNA was cloned into a pGex expression vector. DNA was transformed into Escherichia coli DH5α bacterial cells and grown on LB agar plates with 50 μg/ml ampicillin. One colony was picked and placed in 10 ml Luria–Bertani medium with ampicillin (LB medium) for overnight growth [19]. One ml from the overnight culture was added to one liter of LB medium and grown at 37°C until OD₆₀₀ reached 1.0. Protein production was induced with a final concentration of 1 mM isopropyl β-D-thiogalactopyranoside (Sigma, St. Louis, MO) added to the culture and incubated at 30°C for 4 h. Cells were centrifuged and resuspended in lysis buffer (50 mM Tris pH 8.0, 1 mM EDTA, 1 μM PMSF, 1 mg/ml lysozyme) and sonicated 25 times for 30 s bursts with 1-min pauses between bursts. Triton X-100 was added to a final concentration of 0.1%. Debris was removed by centrifugation. Protein was affinity purified from the bacterial lysate using glutathione-agarose (Sigma) incubated 4 h at 4°C. Protein was eluted from the agarose in a batch format with 10 mM reduced form glutathione (Sigma) under mild conditions [20].

An aliquot of eluted and whole cell lysate material was characterized by western blot analysis. Ten μl of lysate were added to 10 μl of 2× Laemmlli sample buffer and boiled for 30 s. Samples were run on duplicate 12% SDS-PAGE [21]. One gel was stained with Coomassie blue. Proteins on the second gel were transferred to Westman Membrane (Schleicher and Schuell, Keene, NH). Anti p23 monoclonal antibody 7D10 and isotype control monoclonal antibody 5.90.1 were used to incubate the membrane overnight at 4°C [13]. Horseradish peroxidase-linked goat anti-mouse secondary antibody (Cappel #55570, Organon Teknika Corporation, Durham, NC) and ECL detection system (Amersham Pharmacia Biotech) were used to visualize proteins according to manufacturer’s directions.

2.3. Animals

Twenty-three third-trimester Holstein cows were selected on the basis of low serum antibody titers to C. parvum rC7 as determined by ELISA (see below). Nine were not immunized. Fourteen cows were immunized three times at two-week intervals with 300 μg affinity purified rC7 incorporated in monophosphoryl lipid A trehalose dimycolate adjuvant (R-700, Ribi, Hamilton, MT) and injected subcutaneously in the lateral aspect of the neck. First milking colostrum was collected
from each cow at parturition. Colostrum from the nine control cows was pooled and stored in 1500-ml aliquots at \(-80^\circ C\) until thawed for use. Colostrum from the 14 immunized cows was similarly pooled and stored.

Twelve male Holstein calves were obtained at birth from the North Carolina State University, College of Agriculture and Life Sciences, dairy farm. Just prior to parturition, each cow was washed thoroughly and bedded in two feet of fresh straw to minimize environmental contamination to the calf. Calves were transported to the College of Veterinary Medicine within 30 min of birth and housed on straw in a P2 isolation facility. The umbilical cord was dipped in 7% iodine solution, blood was drawn to obtain serum and plasma, rectal body temperature was recorded and 1 ml selenium vitamin E (BO-SE\textsuperscript{1}, Schering-Plough, Kenilworth, NJ) was administered IM. Calves were assigned to colostrum treatment groups by blind code. They were fed 3 l of colostrum within 2 h of birth via gastric intubation. Two l of colostrum were fed at 12 h postpartum from a nipple bottle and 1 l was fed by bottle 24 h postpartum. All colostrum feedings were from a pooled source of frozen and thawed first milking colostrum from the designated treatment group. After 24 h of age, calves were fed antibiotic-free milk replacer at 10% of birth weight, twice daily, for the remainder of the trial.

All calves were infected with \(10^7\) *C. parvum* oocysts in 50 ml colostrum at the 12 h postpartum feeding. Three days post exposure to oocysts, calves were placed in metabolism crates measuring 124 \(\times\) 58 \(\times\) 91 cm (\(l \times w \times h\)) lined with perforated tenderfoot flooring material. Halters and leads were placed on calves and secured to the front of the crate to prevent calves from turning around, while allowing them to stand up, lay down and stretch their limbs. A plastic urine diversionary device was attached approximately 24 cm from the rear of the crate. This urine shunt device was 48 cm long and angled forward from the underside of the crate to divert urine into a collection pan. Feces were collected in a separate rear collection pan. Fecal volume was measured and consistency was evaluated twice daily. Fecal material from each collection was thoroughly mixed in a food processor and a 50 ml representative sample retained at 4°C for future oocyst quantitation. A fecal smear slide was made from each morning sample and examined microscopically after Kinyoun’s acid fast stain to determine presence of oocysts. Rectal body temperature and hydration status were evaluated twice daily. Once diarrhea subsided and oocyst shedding ceased, calves were removed from metabolism crates and returned to pens bedded with straw. Fecal samples from all calves were examined and cultured for known viral and bacterial enteropathogens [4].

### 2.4. ELISA

Serum and colostral antibody levels to *C. parvum* rC7 were estimated by ELISA. Antigen attachment to microtiter plate wells was accomplished by incubating 150 μg rC7 per well at 4°C overnight. Colostral whey was obtained by centrifuging whole colostrum at 28,800 \(\times\) g for 120 min. One hundred μl diluted serum or colostral whey were added to microtiter plate wells previously coated with rC7. Antibody in serum or colostral whey was then detected by adding rabbit-anti bovine Ig H&L conjugated to HRPO (Zymed, S. San Francisco, CA), followed by OPD (Sigma) substrate. The reaction was terminated with 5N H\(_2\)SO\(_4\) and color intensity determined spectrophotometrically.

### 2.5. Fecal oocyst quantitation

Oocysts were quantified in each fecal sample by subjecting a measured fecal volume to centrifugation through a Sheather’s solution gradient as previously described [22]. Oocysts collected from the interface of gradient layers were collected and counted in a hemocytometer at a magnification of \(\times200\). Total oocysts in a given collection were calculated by multiplying the number of oocysts per ml times the volume of the collection.

### 2.6. Statistical analysis

Data were analyzed by analysis of variance using the General Linear Models procedure of SAS [23]. A probability level of \(p < 0.05\) was considered significant.

### 3. Results

#### 3.1. Affinity-isolated rC7 antigen

Fig. 1 depicts the appearance of rC7 in whole bacterial cell lysates and following isolation by affinity chromatography using glutathione S-transferase. The chimeric fusion protein has a molecular mass of approximately 49 kDa. Proteins identified by mAb 7D10 at approximately 44 and 28 kDa represent reduced or degraded products of the recombinant chimeric protein.
In preliminary experiments, adult cattle responded poorly following three subcutaneous injections with 100 μg rC7 in adjuvant. Subsequent trials revealed adequate responses when the antigen dose was increased to 300 μg. Pooled colostrum from immunized cows reacted with rC7 in ELISA at a titer of >1:100,000. The titer of colostrum from control cows was <1:100.

### 3.2. Oocyst excretion in colostrum treated calves

Calves were assigned to treatment groups by blind code. At the completion of the study the code was resolved and it was determined that calves 1, 3, 5, 7, 9 and 11 had received immune colostrum, while calves 2, 4, 6, 8, 10 and 12 were treated with control colostrum. All six calves administered pooled control colostrum developed severe diarrhea and shed an average total of $1.87 \pm 1.66 \times 10^{12}$ *C. parvum* oocysts over the course of the experiment (Fig. 2). The six calves administered pooled immune colostrum shed significantly fewer total oocysts ($3.05 \pm 2.26 \times 10^{9}$, $p < 0.05$) and did not develop diarrhea. Treatment with immune colostrum reduced mean oocyst excretion by 2.79 log$_{10}$ (99.8%).

### 3.3. Signs of disease in colostrum treated calves

All six calves administered pooled control colostrum developed severe diarrhea (mean total fecal volume = 8447 ± 5600 ml) (Fig. 3). None of the six calves administered pooled immune colostrum developed diarrhea (mean total fecal volume = 740 ± 750 ml, $p < 0.05$). Group differences in daily fecal volume were significant on days 4, 5, 6 and 8 postinfection with *C. parvum* oocysts (Fig. 4). None of the calves treated with immune colostrum was febrile or disinter-

![Fig. 1. SDS-PAGE characterization of *C. parvum* rC7 obtained from *E. coli* DH5α bacterial cells. (a) Western blot analysis of bacterial lysate (lanes 1 and 3) and affinity-purified rC7 (lanes 2 and 4) probed with anti-p23 mAb 7D10 (lanes 3 and 4) and isotype control mAb (lanes 1 and 2). (b) Coomassie blue stained gel of affinity-purified rC7 (lane 1) and bacterial lysate (lane 2).](image1)

![Fig. 2. Excretion of *C. parvum* oocysts by 12 calves treated with rC7 immune colostrum (hatched bars) or control colostrum (open bars) over the course of the experiment. Mean and one standard deviation of each group is indicated with the ■ and □, respectively. Group means are significantly different * (p < 0.05).](image2)
ested in eating. In contrast, four of the six calves given pooled control colostrum were noticeably depressed following onset of diarrhea. Calf 6 became recumbent, did not respond to administration of oral electrolyte solution and died 7.5 days postinfection. No bovine enteropathogens, other than \textit{C. parvum}, were detected in fecal samples from calf 6 or the other 11 calves in the study.

4. Discussion

In this study we demonstrated that immune bovine colostrum, induced by immunization of late-gestation cows with \textit{C. parvum} recombinant protein rC7, provided substantial protection to calves challenged with $10^7$ \textit{C. parvum} oocysts. Protection was evidenced by absence of diarrhea and significant reduction
(p < 0.05) in oocyst shedding by calves treated with rC7-immune as opposed to control bovine colostrum. Calves fed rC7-immune colostrum shed an average of 3.05 ± 2.26 × 10^9 oocysts, compared to 1.87 ± 1.66 × 10^12 C. parvum oocysts in calves treated with control colostrum. The absence of diarrhea and 2.79 log_10 (99.8%) reduction in oocyst excretion indicates that immune bovine colostrum induced by immunization with C. parvum recombinant protein rC7 ameliorated clinical manifestations of cryptosporidiosis and reduced the potential for environmental contamination by an infectious agent of major public health significance [1, 6, 24, 25].

This study was designed to minimize the number of variables influencing the outcome. Our intent was to compare a standardized source of immune colostrum to a standardized source of control colostrum. All donor cows were initially tested to verify minimal preexisting antibody titer to rC7, a recombinant protein containing the 101 C terminal amino acids of p23, a surface protein of sporozoites and merozoites known to contain at least two neutralization-sensitive epitopes [13]. Immune colostrum donor cows were immunized by a standardized protocol employing affinity-purified rC7. The resulting colostrum was pooled, aliquoted and stored in such a manner that the six calves receiving immune colostrum received the same volume of identical colostrum at consistent times following birth. In similar manner, control colostrum was pooled, aliquoted and stored so that recipient calves received consistent product. All calves were obtained at birth from the same source and were managed consistently. The C. parvum oocysts were obtained from a single source. While different lots of oocysts were used during the course of the study, all demonstrated >90% excystation at the time of administration. Although not known until study completion, calves were regularly assigned to alternate treatment groups. This further reduced the impact of subtle differences in oocyst viability on the response of infected calves. The only apparent variable in the study was immunization or lack of immunization of colostrum donor cows with C. parvum rC7 in adjuvant.

Although the mechanism(s) by which rC7-immune bovine colostrum protected calves against cryptosporidiosis and reduced oocyst excretion were not determined, neutralizing antibodies are the most likely contributor to protection. C. parvum rC7 contains at least two neutralization-sensitive epitopes. One of these epitopes is linear, QDKPAD, and occurs twice in the recombinant protein employed for immunization of rC7-immune colostrum donor cows [13]. The other epitope appears to be conformationally-determined and its copy number within rC7 is unknown. Murine monoclonal antibodies directed to these two epitopes neutralize sporozoite infectivity and substantially reduce infection of neonatal mice challenged orally with C. parvum oocysts [13, 26, 27]. Cattle immunized with other C. parvum antigens have been shown to produce colostrum containing immunoglobulins that neutralize C. parvum life cycle stages [28–35].

Bovine colostrum has been used with variable success to treat immunodeficient humans persistently infected with C. parvum [36–40]. Studies with neonatal and immunodeficient mice have shown that immune bovine colostrum reduces C. parvum infection or parasite load [28–30, 32, 41]. In some instances, protection has been linked to antibodies within the colostrum preparations [29, 30, 34].

Previous attempts to protect calves against cryptosporidiosis with immune bovine colostrum yielded inconsistent results [28, 31, 42–44]. The C. parvum antigen preparations in those studies were intact oocysts, solubilized oocysts, or solubilized sporozoites. In the current study we employed affinity-purified recombinant protein containing known neutralization-sensitive epitopes. Use of this recombinant protein may increase the likelihood of immune responses targeted to epitopes relevant in protection against cryptosporidiosis.

The results of this study demonstrate that immunization of cattle with a characterized recombinant protein induced immune colostrum that protected calves against disease and significantly reduced oocyst shedding following challenge with C. parvum oocysts. The use of this and other recombinant proteins containing neutralization-sensitive epitopes may result in immune products of value in preventing or treating cryptosporidiosis in humans, calves and other animals.

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