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Resistance of Calves to *Cryptosporidium parvum*: Effects of Age and Previous Exposure

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Cryptosporidium parvum **is a coccidian parasite that causes diarrheal disease in many vertebrate species, including young (≤1 month old) calves. Older calves and adult cattle are resistant to infection. In this study, newborn calves were raised in isolation from *C. parvum* for 1 week to 3 months before experimental challenge with the parasite. Calves orally challenged with *C. parvum* at 1 week of age shed oocysts in their feces and had diarrhea after challenge exposure. When these calves were rechallenged at 1 and 3 months of age, they neither shed oocysts nor had diarrhea. There was no significant increase in the mean anticryptosporidium enzyme-linked immunosorbent assay serum antibody titer in these calves following any of the challenge exposures. Calves orally inoculated with *C. parvum* for the first time at 1 month of age shed oocysts, had diarrhea after challenge exposure, and were resistant to rechallenge at 3 months of age. These calves had a twofold increase in serum antibody titer after the first challenge and no increase after the second challenge. Calves orally inoculated with *C. parvum* for the first time at 3 months of age shed oocysts, and two of seven animals had diarrhea. These calves had a 10-fold increase in serum antibody to *C. parvum* after exposure. This study demonstrates that calves raised in isolation from *C. parvum* remain susceptible to challenge until at least 3 months of age. Furthermore, within this time period, initial exposure and recovery renders calves resistant to further challenge with the parasite. The data also suggest that exposure of young calves to *C. parvum* may inhibit the development of a serum antibody response to the parasite.**

Cryptosporidium parvum is a coccidian parasite of the alimentary and respiratory mucosa in numerous vertebrate species (8, 22). Cryptosporidia are frequently associated with enteric disease in calves, humans, and other species (17). *C. parvum* can be readily transmitted between host species, and cryptosporidiosis is a zoonotic disease (5, 15). The infection is usually mild and self-limiting in animals or humans with a normal immune system but can be chronic and life threatening in immunocompromised individuals (3, 17).

Although young calves (up to 1 month of age) are commonly infected with *C. parvum*, infection is seldom seen in older calves and adult cattle (4, 8). This apparent age-related resistance is also seen in other species. Human cryptosporidiosis is more common in children than in adults (8, 20). Infant laboratory mice are susceptible to *C. parvum* infection, while adults are resistant even in the absence of previous exposure to the parasite (7, 9, 12, 19). This age-related resistance in mice may be partly due to the acquisition of mature intestinal flora, since adult germfree mice are susceptible to infection with *C. parvum* (9).

In view of the economic importance and zoonotic potential of cryptosporidiosis in calves, we have studied the immune response of calves to *C. parvum*. We report here that all calves first exposed to the parasite at either 1 week or 1 month of age shed oocysts and had diarrhea. These calves were resistant to subsequent challenge with *C. parvum* but had only a twofold or less increase in serum antibody to the parasite following any of the challenges. Calves first exposed at 3 months of age shed oocysts, and two of seven animals had diarrhea. These calves had a 10-fold increase in serum antibody to *C. parvum* following challenge.

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MATERIALS AND METHODS

Calves. Nineteen Holstein or Hereford-Angus crossbred calves were collected at birth on a sterile sheet, handled with sterile gloves, and taken to clean isolation rooms. Within 3 h of birth, each calf was bottle fed 1 liter of colostrum which had been previously collected from several normal cows, pooled, sterilized with beta-propiolactone, and stored frozen until use. This colostrum contained anticryptosporidial antibody as determined by indirect fluorescent-antibody testing (14). For subsequent feedings, calves were given milk replacer (Land O'Lakes Corp., Arden Hills, Minn.) twice daily for 2.5 months in accordance with the recommendations of the manufacturer. Starting at 2 weeks of age, calves were offered a pelleted concentrate (Ralston Purina Co., St. Louis, Mo.), and starting at 4 weeks, the calves were offered hay cubes. The calves were weaned to a solid diet at 2.5 months of age.

Calves were housed in isolation rooms (one calf per room) until just before they were inoculated with cryptosporidia. To prevent accidental contamination of the calves with *C. parvum*, personnel showered and changed clothes before entering the isolation rooms. Fomites were thoroughly washed, exposed to hot water (58°C), and allowed to dry for 2 days or sterilized before they were introduced into the isolation rooms. Milk replacer, starter pellets, and hay cubes were frozen and thawed twice before being fed to calves.

Challenge inoculation. Calves to be challenged with *C. parvum* were moved to a separate building just before challenge. The challenge inoculum consisted of 10^7 purified *C. parvum* oocysts per calf prepared as described previously (16). Calves <3 months old were inoculated by mixing the oocysts with milk replacer given at a regular feeding. Calves

TABLE 1. Schedule of challenge inoculation of calves with *C. parvum*

Group	Calf	Challenged at age:			
		<1 wk	1 mo	3 mo	6 mo
1	821	X	X		
	824	X	X		
	241	X	X		
	242	X	X		
	101	X	X	X	
	102	X	X	X	
2	823		X		
	825		X		
	243		X		
	244		X		
	104		X	X	
	105		X	X	
3	822			X	X
	826			X	X
	106			X	
	107			X	
	108			X	
	209			X	
	210			X	

>3 months old were inoculated by mixing the oocysts in drinking water.

The challenge-inoculation schedule is presented in Table 1. Group 1 consisted of six calves (from three separate experiments). These calves were first inoculated with *C. parvum* oocysts before they were 1 week old and rechallenged at 1 month. Two of these calves were rechallenged again at 3 months of age. Group 2 consisted of six calves (from three experiments) that were first inoculated when they were 1 month old. Two of these calves were rechallenged at 3 months. Group 3 consisted of seven calves (from two experiments) that were first inoculated when they were 3 months old. Two of these calves were rechallenged at 6 months.

Monitoring of calves. Fecal samples were collected from each calf three times weekly throughout the experiments and daily for 10 days after inoculation with *C. parvum*. The consistency of the feces (diarrheic or normal) was recorded. Fecal smears were made and stained with carbol fuchsin (11) and examined microscopically for cryptosporidium oocysts. Selected samples were concentrated by Sheather's flotation method (5), and wet mounts of the concentrate were examined for *C. parvum*. Sera were prepared from blood collected by jugular venipuncture from all calves at 1 week of age, just before challenges with *C. parvum*, and 2 to 3 weeks after challenges.

Antibody determinations. Sera from the calves were tested by an anticryptosporidium enzyme-linked immunosorbent assay as previously described (10). Briefly, samples were added to 96-well, U-bottom microtitration plates (Immulon 2; Dynatech Laboratories, Chantilly, Va.) which had been coated with antigen by incubating 2.5×10^4 disrupted *C. parvum* oocysts per well overnight at 4°C. Twofold dilutions of serum were added to the plates, and they were incubated for 1 h at 37°C. Positive and negative control sera were run with each set of plates. After the wells were rinsed, rabbit anti-bovine immunoglobulin (Dako Corp., Santa Barbara, Calif.) was added to each well, and the plates were incubated for 1 h at 37°C. The plates were rinsed, anti-rabbit immunoglobulin-alkaline phosphatase conjugate (Accurate Chemical, Westbury, N.Y.) was added to each well, and the plates were incubated for 1 h at 37°C. The plates were rinsed, paranitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) was added to each well, and the plates were incubated in the dark for 1 h at 22°C, followed by overnight incubation at 4°C. Plates were read with an automated plate reader (Dynatech MR600). Titration endpoints are presented as the reciprocal of the highest dilution giving an absorbance reading twice that of the average readings for the 1:80 dilution of negative control serum. Geometric mean titers within groups were determined by converting titers to $\log_{10}$. The significance of differences in titers before and after challenges with *C. parvum* was determined by using Student's *t* test.

TABLE 2. Oocyst shedding following challenge of calves with *C. parvum*

Group	No. of calves positive/no. examined at age:			
	<1 wk	1 mo	3 mo	6 mo
1[a]	6/6	0/6	0/2	ND[d]
2[b]	0/6	6/6	0/2	ND
3[c]	0/7	0/7	7/7	0/2

[a] Calves first challenged at <1 week.
[b] Calves first challenged at 1 month.
[c] Calves first challenged at 3 months.
[d] ND, Not done.

RESULTS

Tables 2 and 3 summarize oocyst shedding and diarrhea data, respectively, for calves challenged with *C. parvum* at various ages. None of the calves in the study shed oocysts or had diarrhea prior to challenge inoculation with *C. parvum*.

Group 1 calves. All six calves in group 1, challenged with *C. parvum* before 1 week of age, shed oocysts, with a mean onset of 4.8 days postchallenge and a mean duration of 6.2 days. All six had diarrhea, with a mean onset of 3.8 days postchallenge and a mean duration of 6.7 days. When rechallenged with *C. parvum* at 1 month of age, none of the six calves shed oocysts. One of the six calves had diarrhea for 1 day on day 5 postchallenge. When two of the six calves were challenged a third time at 3 months of age, neither calf shed oocysts or had diarrhea.

Group 2 calves. After initial challenge at 1 month of age, all six calves shed oocysts, with a mean onset of 4.5 days postchallenge and a mean duration of 6.5 days. All six had diarrhea, with a mean onset of 3.8 days postchallenge and a mean duration of 5.7 days. When two of the six calves were rechallenged at 3 months of age, neither calf shed oocysts or had diarrhea.

TABLE 3. Diarrhea in calves following challenge with *C. parvum*

Group	No. of calves positive/no. examined at age:			
	<1 wk	1 mo	3 mo	6 mo
1[a]	6/6	1/6	0/2	ND[d]
2[b]	0/6	6/6	0/2	ND
3[c]	0/7	0/7	2/7	0/2

[a] Calves first challenged at <1 week.
[b] Calves first challenged at 1 month.
[c] Calves first challenged at 3 months.
[d] ND, Not done.

TABLE 4. Anticryptosporidium enzyme-linked immunosorbent assay serum antibody titers in calves challenged with *C. parvum*

Group	Reciprocal of group mean titer					
	<1 wk[a]		1 mo		3 mo	
	Pre[b]	Post[c]	Pre	Post	Pre	Post
1[d]	80	95	95	113	226	160
2[e]	57	40	40	113	226	226
3[f]	121	ND[g]	ND	ND	106	1,114

[a] Age of calves at challenge.
[b] Blood collected just before challenge.
[c] Blood collected 2 to 3 weeks after challenge.
[d] Calves first challenged at <1 week.
[e] Calves first challenged at 1 month.
[f] Calves first challenged at 3 months.
[g] ND, Not done.

Group 3 calves. The seven calves in group 3 were maintained in isolation from *C. parvum* for 3 months and did not shed oocysts or have diarrhea during this time. After challenge at 3 months, all seven calves shed oocysts, with a mean onset of 4.9 days postchallenge and a mean duration of 4.7 days. Two of the seven calves had diarrhea, with a mean onset of 5 days postchallenge and a mean duration of 5 days. When two of the seven calves were rechallenged at 6 months of age, neither calf shed oocysts or had diarrhea.

Serology. Table 4 summarizes the enzyme-linked immunosorbent assay anticryptosporidium antibody titers detected in serum samples from 13 calves collected at various times before and after challenge with *C. parvum*. The mean anticryptosporidium antibody titer in sera from calves challenged for the first time at 1 week of age (group 1) did not show a statistically significant increase following any of the challenges. The mean titer in sera from calves challenged for the first time at 1 month of age (group 2) increased twofold following challenge (significant at $P \leq 0.05$) but did not increase after rechallenge at 3 months. The mean antibody titer in sera from calves challenged for the first time at 3 months of age (group 3) increased 10-fold following challenge (significant at $P \leq 0.01$).

DISCUSSION

Cryptosporidial infection in calves 1 week to 1 month of age is common but is rarely seen in older animals (4, 8). Since the organism is common in the environment of most calves, it is reasonable to assume that animals are exposed repeatedly to the parasite beginning at birth. Therefore, it is difficult to separate the effects of immunity acquired through natural exposure from the effects of nonimmunological age-related resistance on the ability of older animals to withstand challenge with *C. parvum*. To address this, we tested the susceptibility of calves raised in isolation from *C. parvum* to experimental challenge at 1 week, 1 month, and 3 months of age.

We found that calves raised in isolation were susceptible to infection with *C. parvum* even at 3 months of age. Seven of seven calves first exposed to the parasite at 3 months shed oocysts after challenge. In contrast, none of the four calves that had previous exposure to the parasite shed oocysts following rechallenge at 3 months. These results imply that exposure of calves to *C. parvum* results in specific acquired immunity to the parasite.

The relative importance of age-related resistance and specific acquired immunity to *C. parvum* infection in calves is not known. In mice, exposure to the parasite does not appear necessary for the development of resistance to infection. Mice older than 3 weeks of age are resistant to *C. parvum*, even in the absence of previous exposure (7, 9, 12, 19). This resistance coincides with the development of mature intestinal flora, which in the mouse occurs at about 3 weeks of age (6, 18). We sampled ruminal fluid from five of the group 3 calves (including two calves that had previously received oral inoculations of contents of an adult rumen) when they were 3.5 months of age. These calves (susceptible to primary challenge with *C. parvum* at 3 months of age) had some rumen function as measured by biochemical and microbiologic analyses but not the level typically found in a mature bovine (data not presented). Thus, it remains to be determined whether a calf with mature rumen function but no previous exposure to *C. parvum* would be resistant to challenge with the parasite.

Calves first exposed to *C. parvum* at 1 week or 1 month of age had a twofold or less increase in serum antibody titers to the parasite after initial or subsequent challenges. In contrast, anticryptosporidium serum antibody in calves challenged for the first time at 3 months of age increased 10-fold. One possible explanation for these results is that oral exposure of the young calf to *C. parvum* may induce systemic immunologic tolerance to the parasite. There is ample precedence for the induction of systemic tolerance by oral feeding of protein antigens (21).

The observation that calves in groups 1 and 2 were resistant to rechallenge in the absence of a secondary antibody response to *C. parvum* is not surprising. We and others have reported a lack of correlation between humoral immunity and resistance to infection with *C. parvum* in humans, mice, and calves (1, 2, 16, 23). Similarly, in the present study and a previous study (10), *C. parvum*-specific antibody in normal pooled colostrum did not protect calves from infection. It is likely that T-cell-mediated immunity is necessary for resistance to *C. parvum*, as demonstrated for other protozoan parasites (13).

In summary, this study demonstrates that calves raised in isolation from *C. parvum* remain susceptible to challenge until at least 3 months of age. Furthermore, within this time period, initial exposure and recovery render calves resistant to further challenge with the parasite.

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