INFECTION AND IMMUNITY, Mar. 1988, p. 649–653
0019-9567/88/030649-05$02.00/0

Vol. 56, No. 3

Lacteal Immunity to Enteric Cryptosporidiosis in Mice: Immune Dams Do Not Protect Their Suckling Pups

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Received 3 September 1987/Accepted 27 November 1987

The susceptibilities of passively immunized principal and nonimmunized control suckling mice to orogastric challenge with *Cryptosporidium parvum* oocysts were compared. Principals were suckled by dams that had recovered from *C. parvum* infection. Controls were suckled by dams reared free of *C. parvum* infection. Principals and controls were equally susceptible to challenge. Principals were susceptible even when their dams were hyperimmunized by oral and parenteral booster inoculations with *C. parvum* oocysts. Immune dams produced serum antibody against *C. parvum*, while nonimmune dams did not. Anti-cryptosporidia immunoglobulin G (IgG) and IgA were demonstrated in whey extracted from the stomachs of principals that had suckled immune dams but not in whey extracted from the stomachs of controls. It was concluded that passive lacteal immunity is not an efficient means of protection against cryptosporidiosis in mice. As in other coccidian infections, protective immunity against cryptosporidiosis may depend more on immune cells than on antibody.

Cryptosporidium spp. are protozoan (coccidian) parasites of alimentary and respiratory mucosae in a wide variety of vertebrates. *Cryptosporidium parvum* parasitizes the intestinal tracts of numerous species of mammals, destroys intestinal epithelial cells, is a common cause of diarrhea in cattle (calves), and causes zoonotic disease in humans (1, 4, 6, 14, 15, 24). Enteric cryptosporidiosis in immunologically normal individuals is self-limiting and stimulates the production of antibodies to *C. parvum* (3, 25, 27, 28). However, it may be persistent and life-threatening in humans with immunodeficiencies such as acquired immunodeficiency syndrome and in athymic (nude) mice (6, 8, 17). Calves that recover from infection are resistant to a second challenge with the same strain of *C. parvum* (14). Thus, recovery apparently depends on a specific acquired immune response and conveys protective immunity. Persistence of the disease in nude mice and acquired immunodeficiency syndrome patients suggests that immunity is T lymphocyte (probably the T-helper-inducer subset) dependent. It is not known whether the effector mechanisms of this T-cell-dependent immunity are mediated by antibody, cells, or both.

If antibody protects against cryptosporidiosis, it is reasonable to expect that female mice that have recovered from infection would provide passive lacteal protection to their suckling pups. We report here that dams which recovered from enteric cryptosporidiosis and were hyperimmunized did not protect their suckling mouse pups from experimental cryptosporidiosis even though their milk contained antibody to the parasite.

(This work was presented, in part, at the 10th International Symposium on Intestinal Microecology, Minneapolis, Minn., 2 to 3 October 1985.)

MATERIALS AND METHODS

Mice. Pregnant CF1 outbred (trials 1 to 3) and BALB/c inbred (trials 4 and 5) mice were housed with one mouse per cage, given pelleted food and water ad libitum, and allowed to whelp and suckle their pups. Pups were weaned at 3 weeks of age. Principals and controls were housed in separate rooms after inoculation with *C. parvum*.

***C. parvum*.** Feces containing *C. parvum* oocysts were collected from experimentally infected calves, suspended in 2.5% potassium dichromate, passed through a series of sieves (30), and stored at 4°C for up to 3 months. Oocysts were isolated by layering 25 ml of a fecal dichromate suspension over step gradients made from Sheather sucrose solution. Gradients were composed of 25 ml diluted to a specific gravity of 1.18 g/ml covered by 50 ml at 1.09 g/ml and by 25 ml at 1.02 g/ml. Gradients were centrifuged for 15 min at 900 × *g* in a swinging bucket rotor. The 1.09-g/ml gradient was collected and diluted with Dulbecco phosphate-buffered saline, and oocysts were pelleted by centrifugation at 900 × *g* for 10 min. These oocysts were termed grade A. The bands which formed on either side of the 1.09-g/ml gradient were collected, diluted, and pelleted as described above. These oocysts were termed grade B. Isolated grade A and B oocysts were stored in 2.5% potassium dichromate at 4°C for up to 3 months. Immediately before use, oocysts were washed three times in Dulbecco phosphate-buffered saline to remove potassium dichromate, and numbers were determined by direct counts in a hemacytometer. Grade A oocysts were used for parenteral inoculations because they contained less debris than grade B oocysts. Grade B oocysts were used for intragastric and oral inoculations.

Experimental design. Recovered immune female mice (principal dams) were produced by intragastric inoculation of all pups in a litter with 10^6 oocysts per pup at 5 to 7 days of age. Infection was confirmed by euthanatizing one or two pups per litter 5 to 7 days after inoculation and demonstrating *C. parvum* oocysts by microscopic examination of carbolfuchsin-stained smears of their colon contents (8). The infection was allowed to run its course in the remaining pups, and at 55 to 60 days of age, the females were bred to nonlittermate males. In some trials (see below), recovered

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immune females were given oral and parenteral booster inoculations with oocysts. Nonimmune females (control dams) were never inoculated; one or two of their littermates were euthanatized at 10 to 14 days of age and examined for *C. parvum* oocysts in colon contents (none were found). Control dams were also bred to nonlittermate males at 55 to 60 days of age.

In trials 2 to 5 (see Table 2), recovered immune females were given oral booster inoculations 1 week before they were bred. For this purpose, they were deprived of water for 1 day and then each was allowed to drink (from a hand-held syringe) 10^7 *C. parvum* oocysts in water. In trials 4 and 5, immunizations included parenteral booster inoculations with *C. parvum* oocysts (10^7/ml). In trial 4, parenteral booster inoculations were given when the females were 1 month old, again at 55 to 60 days of age, and a third time 10 days after breeding. Each mouse was inoculated at three different sites (subcutaneously [0.1 ml], intramuscularly [0.1 ml], and intradermally in the footpad [0.05 ml]), and the inoculations were repeated (three sites per mouse) at each of the three parenteral booster inoculations. In trial 5, the oocysts were mixed with an equal volume of Freund complete adjuvant, and 0.25 ml of the mixture was inoculated intraperitoneally into each mouse at 1 month of age. Ten days after breeding (10 to 12 days prepartum), trial 5 mice were given a second parenteral booster inoculation like that given in trial 4 (three sites per mouse).

Pups born to and suckling either principal or control dams were challenged at 5 to 7 days of age by intragastric inoculation with various doses (see Tables 1 and 2) of *C. parvum* oocysts. Colon contents of the pups were examined for oocysts at necropsy 5 to 7 days after inoculation unless stated otherwise below. In some experiments (see Table 2), sections of ileum were collected and examined histologically for *C. parvum*.

Antibody. In trials 3 through 5, sera were collected from dams, and gastric milk curd extract (whey) was collected from pups at necropsy and tested for antibody. Pups were removed from their dams for 4 h, allowed to suckle their dams for 1 h, and then euthanatized. Milk curds were removed from the stomachs of the pups and emulsified in 2 volumes of saline. The emulsions from littermates were pooled and centrifuged (20,000 × *g*) for 1 h at 4°C. The supernatant fluids (whey) were stored at −70°C until tested for antibody.

The enzyme-linked immunosorbent assay, described previously (28), was modified to detect mouse antibody by replacing the alkaline phosphatase-conjugated goat antibody to human immunoglobulin G (IgG) or IgM with peroxidase-conjugated goat antibody to mouse immunoglobulin (Kirkegaard and Perry, Gaithersburg, Md.). Serum samples were tested at a 1:10 dilution, and whey samples were tested at a 1:4 dilution. Some whey samples (see Table 4) were also tested separately for anti-*Cryptosporidium* IgA and IgG by using commercially available (Kirkegaard and Perry) immunoglobulin class-specific conjugates (specificity not verified).

RESULTS

In the first trial, pups born to recovered and control dams were each challenged with 10^6 oocysts. Pups were euthanatized at intervals from 6 to 15 days postinoculation (PI), and their colon contents were examined for *C. parvum* oocysts in their colon contents (Table 1). All pups examined on days 6 and 9 were infected. The proportion of infected pups decreased after day 9 PI, and on day 15 PI oocysts were detected in only three of six control (suckling nonimmunized dams) and none of six principal (suckling recovered dams) pups. None of the pups in this trial or in subsequent trials developed diarrhea. Because trial 1 results were consistent with a low level of protection, in all subsequent trials, recovered immune dams were given an oral booster dose of oocysts and the dose used to challenge the pups was reduced.

TABLE 1. *C. parvum* infection in mouse pups suckling control dams or dams that recovered from neonatal *C. parvum* infection (trial 1)

Dam group (no.)	No. of pups[a] at day (PI):			
	6	9	12	15
Control (4)	7/7	7/7	6/9	3/6
Recovered (5)	10/10	10/10	2/8	0/6

[a] Expressed as the number with oocysts in the colon contents per the number examined. Every pup was inoculated with 10^6 oocysts. One to three pups per litter were euthanatized and examined on each day listed.

Tests on the infectivity of *C. parvum* oocysts for 5- to 7-day-old control pups (data not shown) indicated that the 50% infectious dose was $<10^2$ oocysts per pup. In the second trial, pups were challenged with 10^3 oocysts and examined five to seven days PI. All of the controls and all but one of the principals were infected (Table 2). Furthermore, the numbers of oocysts in smears of colon contents (Table 2) from principals and controls were similar. When the challenge was reduced to 10^2 oocysts in trial 3 (Table 2), there was still no convincing evidence that principal dams protected their pups. Trial 3 controls did not have detectable levels of antibody to *C. parvum* in either serum from dams or whey from pups. Principals had detectable levels of antibody in the serum from dams but not in the whey extracted from the stomachs of the pups (Table 3). The immunization regimen for the remaining trials was intensified by giving parenteral booster inoculations to the recovered orally boosted females.

The principal dams in trial 4 received three parenteral boosters and had detectable antibody in both serum and whey (Table 3). However, their suckling pups became infected after challenge (Table 2). Furthermore, the numbers of organisms in the intestines (Table 2) of principals and controls appeared to be comparable. In trial 5 (parenteral booster in adjuvant), the level of whey antibody detected was comparable to that in trial 4 (Table 3). The first principal and control litters in trial 5 were challenged with 10^2 oocysts per pup, and none of the pups in either group became infected (data not shown). We suspected that the oocysts had lost infectivity during storage. Therefore, the remaining litters in trial 5 were challenged with 10^5 oocysts per pup. Even though the challenge was again less than a 100% infectious dose, pups suckling the hyperimmunized dams were just as susceptible as those suckling control dams (Table 2).

Tests for anti-*C. parvum* IgG and IgA indicated that immunization stimulated production of both specific IgG and specific IgA in the whey of principals in trials 4 and 5 (Table 4).

DISCUSSION

Mouse pups suckling dams that had recovered from cryptosporidiosis or that had recovered and been orally and parenterally hyperimmunized were not protected against

TABLE 2. Effect of oral immunization of female mice on the response of their suckling pups to inoculation with *C. parvum* oocysts[a]

Trial	Dam (no.)	Parenteral booster	No. of oocysts inoculated	Infected pups[b]			
				Ileum[c]		Colon[d]	
				No.	Intensity	No.	Intensity
2	Control (8)	0	10^3			52/52	1.8
	Immune (7)	0				50/51	1.9
3	Control (7)	0	10^2			47/52	1.9
	Immune (7)	0				40/52	1.2
4	Control (6)	0	10^2	6/25	0.5	5/25	0.4
	Immune (4)	+		9/22	0.8	10/22	0.5
5	Control (4)	0	10^5	14/16	1.5	13/16	1.2
	Immune (3)	+-Adjuvant		11/11	1.8	10/11	1.6

[a] Immune dams recovered from *C. parvum* infection were given an oral booster with *C. parvum* oocysts 1 month later. Those in trials 4 and 5 were also vaccinated parenterally with *C. parvum*. Control dams were not exposed to *C. parvum* before inoculation of their pups.

[b] At necropsy 5 to 7 days after inoculation.

[c] Determined by histologic examination. The number is expressed as the number infected per the number examined. Intensity is the mean score, graded as 0 (*C. parvum* not detected), 1 (few *C. parvum*), or 2 (many *C. parvum*).

[d] *C. parvum* oocysts detected in smears of colon contents. The number is expressed as the number positive per the number examined. Intensity is the mean number of oocysts per microscopic field (magnification, ×500): 0, none; 1, >0 to 1; 2, 1 to 5; 3, 6 to 20; 4, >20.

infection with *C. parvum*. The first trial suggested a low level of protection detected during resolution of the infection. It is conceivable that protection via lacteal immunity could be reflected as enhanced resolution and not detected during the acute phase of infection. We considered this to be unlikely and, in subsequent trials, assumed that if protection existed, it would be detectable during the acute phase of infection after lower challenge doses. Trials to determine if immunity leads to enhanced resolution in this model are warranted. Our results agree with those of others who also found that recovered mice did not protect their suckling pups against *C. parvum* infection (M. J. Arrowood and C. R. Sterling, Abstr. 61st Annu. Meet. Am. Soc. Parasitol. 1986, abstr. no. 91, p. 54). They are also consistent with the observations that (i) nude mice (28 loc. cit.) and acquired immunodeficiency syndrome patients (3, 27) produce antibody against *C. parvum* but remain persistently infected with the organism and (ii) oral treatment of human cryptosporidiosis with anti-cryptosporidia antibody in bovine colostrum was not effective (22). Whey extracted from the stomachs of the susceptible passively immunized pups in at least two of our trials contained anti-*C. parvum* antibodies of the IgG and IgA isotypes. We were interested in IgA because of its suitability for protection against other enteric infections and because the reduced ability of nude mice to produce IgA (7) could theoretically account for their inability to recover from infection. The test for whey antibody detected only antibody trapped in the stomach within the semisolid milk curd and surviving up to an hour of gastric digestion. We tested for residual antibody extractable from milk curds because of the technical problems associated with collecting intestinal contents from pups or milk from dams.

Our results do not exclude the possibility that protection might be demonstrable with higher levels or different isotypes of antibody than those attained here. Nor do they exclude the possibility that critical antigens may have been lacking from our booster immunizations. Neither the life cycle stages nor the antigens involved in protective immunity to *C. parvum* are known. We used recovery from infection for primary immunization so that immune dams would be exposed via the natural mucosal route to all stages of the parasite. The booster inoculations contained oocyst and sporozoite antigens. Sporozoites are theoretically an attractive stage for attack in antibody-mediated protection. Hyperimmune serum with a high titer of *C. parvum* antibody neutralized the infectivity of sporozoites, while preimmune serum with a low titer of *C. parvum* antibody did not (18). Excystation would expose the sporozoites to lacteal antibody in the intestinal lumen before they contacted epithelial cell microvilli and initiated infection. Protection against malaria can be mediated by antibody directed against sporozoites (5). Lacteal immunity protects against the localized enteric infections caused by *Giardia* spp., enterotoxigenic *Escherichia coli*, rotaviruses, and coronaviruses (2, 16, 19,

TABLE 3. Effect of immunization of female mice on *C. parvum* antibody in serum and whey[a]

Trial	Group	Optical density[b]				
		Serum	Whey[c]	PBS	Standard mouse serum	
					Negative	Positive
3	Control	0.45 ± 0.01	0.22 ± 0.02	0.49	0.78	1.46
	Immune	1.09 ± 0.06	0.29 ± 0.06			
4	Control	0.42 ± 0.01	0.24 ± 0.01	ND	0.62	1.54
	Immune	1.12 ± 0.02	0.96 ± 0.06			
5	Control	ND	0.23 ± 0.02	ND	0.61	1.91
	Immune	ND	0.94 ± 0.03			

[a] See Table 2 and footnotes for immunization schedule and number of mice involved.

[b] For serum and whey, the optical density is expressed as the mean ± the standard error of the mean optical density at 405 nm produced in an enzyme-linked immunosorbent assay for antibody (mouse immunoglobulin) to *C. parvum*. Phosphate-buffered saline (PBS) and mouse serum previously shown to be free of (negative) or to contain (positive) antibody were used as standards. ND, Not done.

[c] Stomach contents from pups that had suckled were extracted with saline. The resulting whey extracts from littermates were pooled and tested for antibody.

TABLE 4. Effects of immunization of female mice on anti-*C. parvum* IgG and IgA in whey[a]

Trial	Group	No. of samples[b]	Mean optical density (range)[c]	
			IgG	IgA
4	Control	2	0.12	0.13 (0.11–0.14)
	Immune	4	1.03 (0.96–1.15)	0.59 (0.36–0.72)
5	Control	2	0.07	0.08
	Immune	5	0.89 (0.80–1.02)	0.99 (0.76–1.28)

[a] See Table 2 and footnote *a* for immunization schedule.
[b] Stomach contents from pups that had suckled were extracted with saline. The resulting whey extracts from littermates were pooled into a single sample.
[c] Optical density at 405 nm in enzyme-linked immunosorbent assays.

20). However, our data suggest that lacteal immunity provides little or no protection against cryptosporidiosis in mice.

There is indirect and conflicting evidence regarding protection of human infants against cryptosporidiosis via lacteal immunity (11, 12, 29). Hyperimmune bovine colostrum was reported to be therapeutically effective in a persistently infected human and to neutralize the infectivity of *C. parvum* oocysts for infant mice (26). These suggestions of protective lacteal immunity need confirmation. Lacteal immunity against cryptosporidiosis may be more effective in other species than in mice.

In ruminants, passive lacteal immunity is transferred to the neonate almost totally as IgG in colostrum (20). The immediate postcolostral period, i.e., the second and third weeks after birth, is the time of the greatest incidence of cryptosporidiosis as well as of rotavirus and coronavirus infections in calves (4, 6, 14, 19, 20, 23). On the other hand, sucklings of nonruminant species ingest comparatively high levels of IgA antibody in postcolostral milk, and the greatest prevalence of such infections tends to be delayed until after weaning. The comparatively short-lived enteric protection by lacteal antibody in ruminants could hypothetically explain why cryptosporidiosis is more prevalent among calves than among sucklings of nonruminant species (1, 4, 14, 21, 23, 24).

On the other hand, protective immunity against most coccidian parasites depends primarily on cell-mediated immune responses (10, 13). Even in malaria, in which the protective effects of antibody have been convincingly demonstrated, immune effector cells are required for solid protection (5). Nonimmunoglobulin products of immune cells (cytokines) probably contribute to protective immunity against malaria (9). Thus, in view of the lack of demonstrable lacteal antibody-mediated protection in mice, we suspect that antibodies are not efficient mediators of protection against *C. parvum* infection. It would be useful to determine whether protection can be transferred adoptively by immune cells or their cytokines. Furthermore, definitive studies are needed on the role of antibody in protection of humans and calves against *C. parvum* infection.

ACKNOWLEDGMENT

This work was conducted with the technical assistance of S. M. Skartvedt, B. A. Pesch, and G. D. Fry.

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