Passive Immunity to Bovine Rotavirus Infection Associated with Transfer of Serum Antibody into the Intestinal Lumen

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The effect of circulating passive antibody on immunity to bovine rotavirus infections in neonatal calves was investigated. In the first experiment, rotavirus antibody titers in the small intestinal lumina of 5- and 10-day-old calves with a wide range of serum rotavirus antibody titers were determined. Neutralizing antibody was present in the small intestinal lumina in titers that correlated with the calves' serum titers ($r = +0.84$, $P < 0.01$). Immunoglobulin G1 was the predominant isotype of intestinal luminal rotavirus antibody. Calves not fed colostrum during the absorptive period lacked rotavirus antibody in circulation and in the intestinal lumen at 7 days of age, even when they were fed large volumes of colostrum with a high rotavirus antibody titer at 48 h after birth. Therefore, rotavirus antibody is not retained in the intestinal lumen for 5 days following a colostrum meal, and the luminal antibody in the 5- and 10-day-old seropositive calves were probably derived from circulating antibody. In a second experiment, calves were passively immunized by subcutaneous injection of colostral whey with a high immunoglobulin G1 rotavirus antibody titer and challenged with virulent bovine rotavirus 48 h later. The passively immunized calves were protected from rotavirus infection and diarrhea compared with calves with comparable serum immunoglobulin concentrations but with lower serum rotavirus antibody titers. The results of these experiments indicate that circulating immunoglobulin G1 antibody appears in the gastrointestinal tract of neonatal calves and that circulating rotavirus antibody can prevent infection and diarrhea after rotavirus challenge.

Rotaviruses cause neonatal diarrhea in many species and are one of the most important agents of neonatal diarrhea in calves (22, 39). Because of the ubiquity of rotaviruses in cattle populations, newborn calves that ingest colostrum usually have circulating anti-rotavirus antibody yet remain susceptible to infection with rotavirus in the first few weeks of life (24, 28, 41). As a result of the failure of circulating antibody to protect calves, the general focus of research on passive immunity to bovine rotavirus has been on antibody bathing the intestinal lumen, and in particular on antibody secreted in the milk (8). Calves can be protected from rotavirus infection and diarrhea by supplementation of their milk diet with rotavirus antibody (6, 29, 30, 33, 40). However, immunization protocols designed to elevate and prolong antibody secretion into cow's milk have only partially succeeded in reducing calf infection rates (9, 32, 34).

Despite the susceptibility of seropositive neonatal calves to rotavirus infections, we decided to investigate the ability of high serum antibody concentrations to influence enteric infections. There are data to show that circulating antibody enters the intestinal lumen. (i) Intravenously injected radiolabeled immunoglobulin G1 (IgG1) has been detected in the gastrointestinal tract lumen in clinically healthy calves and adult cattle (7, 25, 26). (ii) Increased rotavirus antibody titers were detected in feces of calves with high serum passive rotavirus antibody titers and were suggested to result from serum antibody transferred into the intestinal lumen (30). (iii) Recently we demonstrated the transfer of several grams of IgG1 antibody from the blood into the gastrointestinal tract lumen each day in neonatal calves with high serum immunoglobulin concentrations (1). (iv) There is a body of epidemiologic evidence demonstrating reductions of morbidity and mortality of neonatal diarrhea in calves with high

circulating immunoglobulin concentrations (14). These findings suggested to us that high titers of serum antibody might result in protective amounts of antibody entering the intestinal lumen. This hypothesis is supported by an experiment by Snodgrass and Wells, who found that parenteral injection of immune serum to two lambs resulted in resistance to rotavirus challenge (36).

Based on the hypothesis that transfer of antibody from the bloodstream into the gastrointestinal tract can protect calves from rotavirus infections, experiments were performed to determine (i) whether circulating rotavirus-neutralizing antibody reaches the intestinal lumen in calves, and (ii) whether rotavirus antibody entering the intestinal lumen can protect calves from rotavirus challenge in the absence of lactogenic antibody.

MATERIALS AND METHODS

Experiment 1: rotavirus antibody transferred to the small intestine. (i) Rotavirus antibody measurement. Tissue culture-adapted bovine rotavirus (Lincoln NCDV strain) was obtained from L. Saif (Ohio Agricultural Research and Development Center, Wooster, Ohio). The virus was propagated in MA-104 cells by using trypsin pretreatment (38). Neutralizing rotavirus antibody titers of colostrum, serum, and small intestinal contents were measured using a plaque reduction assay on MA-104 cell monolayers in 12-well tissue culture plates (Cluster 12; Costar Inc., Cambridge, Mass.) (31). Isotype-specific rotavirus antibody titers were determined by using an enzyme-linked immunosorbent assay (ELISA) as described by Saif et al. (31) with the following modifications. Tissue culture grown rotavirus was partially purified by centrifugation (50,000 × g maximum, 2 h) and suspension in 0.1 volume of phosphate-buffered saline (PBS), followed by centrifugation into 40% sucrose (50,000 × g, 2 h) and suspension in PBS. The resulting pellet was

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sonicated at 100 W for 2 min, diluted 1/40, aliquoted, and stored at $-20°C$. Mock-infected cells were treated identically to generate control antigen. Virus or control antigen was bound to poly-L-lysine-coated 96-well plates (Immulon I; Dynatech Laboratories, Inc., Alexandria, Va.) by the method of Lansdorp et al. (18). The following reagents were added: (i) serial twofold dilutions of the test sample (primary antibody); (ii) diluted (1/5,000) secondary antibody, consisting of monoclonal antibody specific for bovine immunoglobulin IgA (BIg312D3, obtained from W. C. Davis, Department of Microbiology and Pathology, Washington State University, Pullman) or IgG1 (DAS-16; obtained from A. J. Guidry, Milk Secretion and Mastitis Laboratory, U.S. Department of Agriculture, Beltsville, Md.; see S. Srikumaran et al., CRWAD, Chicago, abstr. 56, 1984); (iii) anti-mouse IgG-horseradish peroxidase conjugate diluted 1/1,000 (anti-mouse IgG; Sigma Chemical Co., St. Louis, Mo.); and (iv) substrate, recrystallized 5-aminosalicylate (10). All dilutions were made in PBS containing 0.2% Tween 20 and 0.1% gelatin. Plates were washed between each step with PBS-Tween 20. All incubations were for 30 min at $37°C$, except the substrate, which was incubated at $22°C$. Each sample was tested in duplicate wells against both test and control antigens. A sample dilution was considered positive when the mean absorbance of the test wells was twice (or greater) that of the control wells and had an absorbance of 0.02 or greater, when read in a microplate ELISA reader (Dynatech Laboratories, Inc.). Each test was accompanied by positive (serum from an immunized cow) and negative (PBS-Tween-gelatin) primary antibody controls. Specificity of monoclonal secondary antibodies was confirmed by ELISA with plates coated with purified bovine IgG1, IgG2, IgM, or IgA.

(ii) Colostral rotavirus antibody. Holstein cows were immunized at 6 and 3 weeks before calving by intramuscular injections of 2 ml of infected tissue culture supernatant containing 2×10^9 50% tissue culture-infective doses of NCDV emulsified in 2 ml of Freund incomplete adjuvant. Rotavirus-neutralizing antibody titers in immunized and nonimmunized cows' colostra were 1:500,000 and 1:12,000, respectively.

(iii) Experimental calves. Twenty-four male Holstein calves born on a commercial dairy were randomly assigned to one of eight groups that varied in three experimental treatments: (i) colostrum volume fed (1 or 3.5 liter), (ii) colostrum source (rotavirus-immunized or nonimmunized dams), and (iii) age at necropsy and sampling (5 or 10 days after colostrum feeding). Calves were fed the appropriate colostrum by 3 h of age and then removed from the farm and maintained in isolation pens on a diet of commercial milk replacer (Suckle; Carnation, Inc, Los Angeles, Calif.) at a rate of 15% of body weight daily, divided into two feedings. This milk replacer does not contain rotavirus neutralizing antibody as detected by the plaque reduction neutralization assay. Immunoglobulin absorption after colostrum feeding was assessed by radial immunodiffusion assay of serum IgG1 concentration from blood samples drawn at 48 h of age (21). Two additional groups of calves were studied: (i) three calves were fed 3.5 liters of rotavirus-immune colostrum at 48 h of age and were sampled 5 days later, and (ii) three other calves were fed no colostrum and were sampled at 2 to 4 days of age. At the end of the experimental period, each calf was anesthetized and exsanguinated 2.5 h after the morning feeding.

(iv) Sample collection. At necropsy, ligatures were placed at the pylorus and ileo-cecal junction and the small intestine was separated from its mesenteric attachments. The entire contents of the small intestinal lumen was collected by gravity flow from a midjejunal incision. Samples were frozen to $-70°C$ immediately after collection.

Hemoglobin concentrations in small intestinal luminal contents were measured to rule out blood contamination of necropsy as a source of intestinal content antibody. The tetramethylbenzidine method used (19) was modified by buffering each sample with 0.1 volume of 2.5 M Tris hydrochloride (pH 7.6). The modified assay was sensitive to 0.05 mg of hemoglobin per ml. Blood contamination was nonsignificant in all samples.

(v) Experimental design. The experimental design was a three-factor, randomized complete block design with 24 experimental calves, 3 calves per treatment group. The effects of colostrum source (nonimmunized or rotavirus-immunized cows), amount of colostrum fed (1 or 3.5 liters), and age of the calf (5 or 10 days) were examined on rotavirus-neutralizing titers of the calves' small intestinal contents. Two control groups were used to determine the rotavirus-neutralizing antibody titers in the small intestinal contents of colostrum-deprived calves and the rotavirus-neutralizing antibody titers remaining in the intestine 5 days after a single colostrum feeding at 48 h of age. Data were analyzed by analysis of variance (37).

Experiment 2: protection from rotavirus infection. (i) Colostral whey. Bovine colostral whey was used as a concentrated source of immunoglobulin for administration to the experimental calves. Colostrum was coagulated with rennin (Sigma), centrifuged for 10 min at $22,000 \times g$, filtered through glass wool, and frozen at $-20°C$ until the day of administration. The whey was then thawed, centrifuged for 120 min at $25,900 \times g$ at $4°C$, and filtered through sterile glass wool, resulting in a sterile, clear, isoosmotic fluid.

(ii) Experimental calves. Male newborn Holstein calves were obtained from a commercial dairy. The calves were housed and fed as in experiment 1 and were harnessed for complete fecal output collection. Each calf received one of three sources of passive antibody on the first day of life: (i) subcutaneous injection of 1.25 liters of purified colostral whey from a rotavirus-immunized cow ($n = 5$), (ii) subcutaneous injection of 1.25 liters of purified colostral whey from a nonimmunized cow ($n = 3$), or (iii) 3 liters of nonimmune colostrum fed in the first 3 h of life ($n = 3$). Immunoglobulin absorption after these treatments was assessed by radial immunodiffusion assay of serum IgG1 concentrations (21). The calves were challenged with rotavirus at 3 days of age, and complete fecal collections were made for 10 days after challenge.

(iii) Rotavirus challenge. A virulent NCDV bovine rotavirus passaged in gnotobiotic calves, obtained from Linda Saif, was given orally (1 ml) to a colostrum-deprived calf at 6 h after birth, and the initial diarrheic feces collected before 30 h of age were aliquoted into 2-ml challenge doses and frozen at $-20°C$. The titer of the challenge was not known, but the ELISA (Rotazyme; Abbot Labs Inc., Chicago, Ill.) score was greater than 20 times that of a cultured NCDV rotavirus containing 2×10^8 50% tissue culture-infective doses per ml. Experimental calves were challenged orally with 1-ml doses at 72 and 96 h of age.

(iv) Fecal examination of diarrhea. Complete fecal collections were made at 12-h intervals for the 10-day period after the first rotavirus challenge for each calf. Each 12-h fecal output was weighed, and samples were collected for rotavirus detection and fecal dry matter determination. Fecal dry matter was measured by weighing each sample before and after drying for 48 h at $50°C$.
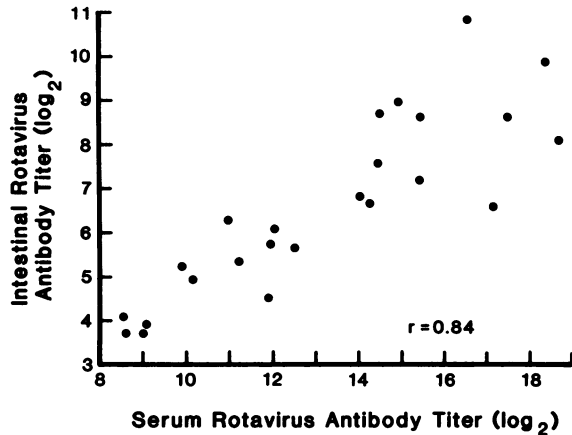
FIG. 1. Serum versus small intestinal rotavirus neutralizing antibody titers in 5- and 10-day-old colostrum-fed calves.

(v) Fecal examination for rotavirus. Each calf was tested for rotavirus shedding (i) by detection of the rotavirus group antigen in each 12-h fecal sample after challenge with a commercial ELISA and (ii) by detection of the rotavirus genome in silver-stained polyacrylamide gel electrophoresis gels of every other 12-h fecal sample after challenge (16).

(vi) Experimental design. Passive immunoglobulin in the form of colostral whey was administered to two groups of calves. The first group was given whey from rotavirus-immune colostrum by subcutaneous injection at 24 h of age, and the second group received colostrum from nonimmunized cows by either colostrum feeding at 3 h of age or by subcutaneous injection of whey at 24 h of age. Infection rates after rotavirus challenge were compared between groups by using the Fisher exact test (37). Mean fecal dry matter scores for each day after challenge were compared between groups by the Mann-Whitney rank-sum test (37).

RESULTS

Experiment 1. To determine whether circulating rotavirus antibody reaches the intestinal lumen, rotavirus antibody titers in the contents of the small intestinal lumina of 5- and 10-day-old seropositive calves were measured. These calves had been given a single feeding of colostrum, pooled from either conventional or rotavirus-immunized cows, at 3 h of age and then had received a maintenance diet of immunoglobulin-free milk replacer. Intestinal lumen antibody titers in the 5- and 10-day-old calves correlated both with serum antibody titers ($r = +0.84$, $P < 0.01$) (Fig. 1) and with serum IgG1 concentrations ($n = +0.759$, $P < 0.01$ for calves fed rotavirus-immune colostrum; $r = +0.690$, $P < 0.05$ for calves fed nonimmune colostrum). There was correlation between IgG1 rotavirus-binding antibody titers and rotavirus-neutralizing antibody titers in the calves' intestinal lumina ($r = +0.796$, $P < 0.01$), whereas IgA rotavirus-binding antibody titers were very low or undetectable and did not correlate with neutralizing titers (Fig. 2). Feeding calves larger volumes of colostrum or colostrum with higher rotavirus antibody titer resulted in higher rotavirus antibody titers in serum and the small intestinal lumen at 5 and 10 days of age ($P < 0.001$). The intestinal titers of 5- and 10-day-old calves fed comparable colostrum volumes did not differ significantly.

Colostral antibody persistent in the intestinal lumen might account for antibody in the intestinal lumen of a 5- or
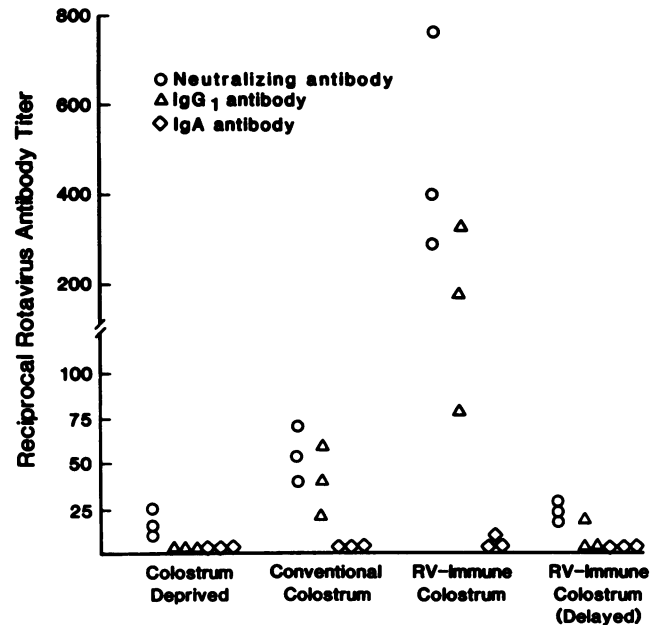

FIG. 2. Neutralizing, IgG1, and IgA rotavirus antibody titers in the small intestinal lumen of calves receiving different colostrum-feeding treatments. The "delayed" group was fed colostrum at 48 h of age and did not absorb immunoglobulin to serum, whereas the other colostrum-fed groups were fed at 3 h of age and absorbed substantial amounts of immunoglobulin to serum. All colostrum-fed calves were sampled at 5 days after colostrum feeding. Three calves were in each group.

10-day-old calf that had been fed colostrum on the first day of life. To test this possibility, 48-h-old calves were fed a large volume of high rotavirus titer colostrum, and the antibody titers in each calf's intestinal lumen was measured after 5 days on an immunoglobulin-free milk replacer diet. Consistent with the inability of the calf to absorb immunoglobulin from colostrum by 48 h of age, these calves remained seronegative. Their intestinal rotavirus antibody titers 5 days after colostrum feeding were no different than those of completely colostrum-deprived calves (Table 1). This indicates that the intestinal rotavirus antibody in 5- and 10-day-old calves was not due to persistence of antibody in the intestinal lumen from the colostrum feeding at 3 h after birth.

Experiment 2. Calves were passively immunized at 24 h of age by subcutaneous injection of 1.25 liters of whey ex-

TABLE 1. Rotavirus-neutralizing antibody in the small intestinal lumen of neonatal calves[a]

Group[b]	Serum IgG1 (mg/ml)	Rotavirus antibody titer ($1/\log_2$)
1	16.6 (1.30)	9.81[a] (0.90)
2	14.7 (3.14)	8.79[a] (0.59)
3	<0.5	4.40[b] (0.19)
4	<0.5	3.91[b] (0.60)

[a] Numbers within a column flanked by different letters are significantly ($P < 0.05$) different means as determined by the least-significant-difference procedure. Results are given as means (standard errors) for three calves.

[b] Group 1 and 2 calves were fed colostrum from rotavirus-immunized cows at 3 h after birth and were sampled at 5 and 10 days of age, respectively. Group 3 calves were fed the same volume and source of colostrum as calves in groups 1 and 2 at 48 h after birth and were sampled 5 days later. Group 4 calves were fed no colostrum and were sampled in the first 2 to 4 days of life.

TABLE 2. Virus shedding and diarrhea in calves
after rotavirus challenge at 48 h of age

Group[a]	Rotavirus antibody titer in serum (1/log$_2$)	Rotavirus infection			Diarrhea[c] (days)
		Rate (no./total)	Incubation[b] (h)	Duration (h)	
Immune	14.85 (0.82)[d]	1/5[e]	72	64	0.10[f] (0.10)
Control	9.10 (0.35)	6/6	32.0 (5.8)	134.8 (7.3)	2.83 (0.99)

[a] Immune calves received a parenteral injection of approximately 75 g of IgG1 derived from colostral whey from rotavirus immunized cattle. Control calves received a similar immunoglobulin mass derived from colostrum from nonimmunized cattle.

[b] Time period from the first oral rotavirus challenge to the first detection of fecal rotavirus shedding.

[c] Days with <15% fecal dry matter.

[d] Standard errors are given within parentheses.

[e] Significantly different from control by the Fisher exact test ($P = 0.015$).

[f] Significantly different from control by the Mann-Whitney rank-sum test ($P = 0.030$).

tracted from the colostrum of rotavirus-immunized cows. These calves received a diet consisting solely of rotavirus-antibody-free artificial milk replacer. Control calves received either subcutaneous injection of whey extracted from the colostrum of a nonimmunized cow or a single feeding of the colostrum of a nonimmunized cow. The serum immunoglobulin concentrations of the two groups did not differ significantly. The serum rotavirus antibody titers of the groups are shown in Table 2.

Subcutaneous rotavirus antibody administration protected calves from rotavirus infection after challenge: four of the five calves in the passively immunized group shed no detectable rotavirus after challenge, whereas all six control calves shed virus ($P = 0.015$) (Table 2). Fecal percent dry matter was significantly higher in the passively immunized calves on days 4 through 8 after challenge ($P < 0.05$).

Rotavirus infections were detected by ELISA and were confirmed by detection of the rotavirus genome in silver-stained electrophoresis gels (16). Of 31 ELISA-positive samples, 29 were positive by electrophoresis; the two exceptions were the two lowest ELISA-positive samples tested. No calf positive by electrophoresis was negative by ELISA. Two rotavirus electropherotypes were detected in the feces of calves that became infected after challenge; one showed the pattern of the challenge rotavirus, and the other differed in the position of genome segments 10 and 11. The second electropherotype was seen in three of the five infected control calves and in the single calf in the immune group that became infected after challenge. The source of the second electropherotype is not known. Parenteral antibody administration apparently protected against both rotavirus electropherotypes.

DISCUSSION

Calves fed colostrum on the first day of life had significant rotavirus-neutralizing antibody titers in their small intestinal lumina 5 and 10 days later. The intestinal antibody titers correlated with the serum antibody titers derived from colostrum and were predominantly of the IgG1 isotype. Intestinal antibody titers were approximately equivalent in 5- and 10-day-old calves, suggesting that antibody transfer to the gastrointestinal tract is a continuing process.

The potential significance of antibody transfer to the intestine was demonstrated by the protection of calves from rotavirus challenge by the administration of colostral immunoglobulin by parenteral injection. This protection was not

due to lactogenic antibody, since the calves received no source of dietary antibody.

It is possible that rotavirus-neutralizing antibody demonstrated in the intestinal lumina of calves in experiment 1 was in part antibody persisting in the intestine from the time of colostrum feeding. Since calves fed colostrum at 2 days of age did not retain rotavirus antibody in the intestinal lumen for 5 days, this possibility would require that IgG1 antibody ingested on the first day of life is protected from intestinal motility and proteolytic activity for at least 5 days, while antibody ingested at 2 days of age is not protected. A simpler explanation is that the rotavirus antibody reached the intestine from the circulation, as was previously shown to occur with anti-DNP antibody (1).

The results of these experiments suggest that passive immunity to calf rotavirus diarrhea could be accomplished by sufficiently elevating calf serum antibody titers. Serum immunoglobulin concentrations and serum rotavirus titers comparable to those of the protected calves in experiment 2 could be attained by feeding calves colostrum with a high rotavirus antibody titer on the first day of life. In previous studies, calves and lambs were generally susceptible to rotavirus infections despite circulating rotavirus antibody (24, 28, 35, 41), but those with high serum immunoglobulin concentrations were relatively resistant to diarrhea associated with rotavirus infections (23, 24, 36). The decrease in clinical signs associated with high serum passive immunoglobulin concentrations in those studies and the protection from rotavirus infections associated with high serum rotavirus antibody titers in the present study are consistent with a protective role for blood antibody transferred to the intestine. The transfer of circulating antibody into the gastrointestinal tract could be the mechanism that results in the decreased diarrheal disease morbidity and mortality in neonatal calves with high passive serum immunoglobulin concentrations, which has been observed in a number of epidemiologic studies (2–5, 11–13, 15, 17, 20, 21, 23, 27).

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