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Immunity to Rotavirus in Conventional Neonatal Calves

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The local and systemic humoral immune responses to rotavirus were studied in six conventional neonatal calves. Attenuated bovine rotavirus was administered either orally or directly into an isolated intestinal loop. The parameters monitored were neutralizing rotavirus antibody in serum, immunofluorescent and neutralizing rotavirus antibody in intestinal loop washings, and rotavirus antibody-producing cells in intestinal mucosa. An antibody response was observed in the serum and intestinal secretions from one calf only. Viral replication was not detected in the isolated intestinal loop. Rotavirus antibody-producing cells were found in the intestinal mucosa of five calves. Double staining revealed that most of these cells produced antibody of the immunoglobulin A class. The conclusions were: (i) a previously described system to detect rotavirus antibody-producing cells can be used to study immune responses in neonatal calves, (ii) the class or subclass of antibody in rotavirus antibody-producing cells can be determined by double immunofluorescent staining, (iii) neonatal calves respond to rotavirus inoculation with a local immunoglobulin A response, and (iv) most of the rotavirus antibody-producing cells are located in the mucosa of the proximal small intestine.

Rotaviruses are a common cause of diarrhea in neonatal animals and human infants (8, 17). Most studies on immunity to rotaviruses have been concerned with passively acquired antibody and the effects of local versus systemic antibody.

Field observations of neonatal calves indicate that circulating rotavirus antibody is ineffective in providing resistance to rotaviral diarrhea (20, 35, 36, 41). Lambs fed colostrum containing rotavirus antibody on day 1 of life are susceptible to rotaviral diarrhea when challenged on day 2. However, lambs fed colostrum through the first 4 days of life and inoculated on day 2 are resistant to infection and diarrhea. Comparable serum antibody titers are present in both groups (29). In addition, lambs fed colostrum containing rotavirus antibody at a time when the antibodies are not absorbed from the intestine lack serum antibody to rotavirus but resist rotavirus challenge (30). Thus, it appears that antibody needs to be present in the gut lumen for effective protection. Serum antibody levels are not a good indicator of resistance to infection. Studies in humans (15, 21, 27, 32), calves (37, 40, 41), and lambs (31, 34) support these conclusions.

Studies on active immunity to rotaviruses are few. Calves inoculated with attenuated bovine rotavirus in utero (42) or shortly after birth (19, 38) are protected against subsequent challenge with virulent virus. Experiments with rotavirus-infected piglets suggest that rotavirus antibody activity in feces is essentially due to immunoglobulin A (IgA) (7).

The purpose of this study was to examine the local and systemic humoral immune response to rotavirus in neonatal calves.

MATERIALS AND METHODS

Virus. An attenuated tissue culture-adapted bovine rotavirus was used (Norden Laboratories, Lincoln, Nebr.). This virus is used in commercial vaccine production.

Cell cultures. Bovine rotavirus was propagated in two cell types. MA-104, an embryonic, rhesus macaque kidney cell line, was obtained from M. A. Bioproducts, Walkersville, Md., and second-passage bovine embryonic kidney cells were obtained from Norden Laboratories. Cell cultures were grown in either Eagle basal medium or Leibovitz L15 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Animals. Male Holstein-Friesian calves were obtained within 24 h after birth. Calves were allowed to nurse once to obtain colostral antibodies from their dams. The calves were housed in individual, thoroughly disinfected rooms. They were fed autoclaved whole milk at a rate of 1 pint (0.473 liter) of milk per 10 pounds (4.54 kg) of body weight per day administered in three or four daily feedings. Water was given ad libitum.

Experimental surgery. An isolated intestinal or Thiry-Vella loop was established surgically in each

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calf (16). A surgical incision was made through the abdominal wall in the region of the right paralumbar fossa. The ileocecal junction was then identified. The small intestine was transected at 2 m and again at 3 m proximal to this point. The ends of the central, isolated, 1-m segment were brought to the surface through stab incisions in the flank and sutured to the skin. In this manner, a U-shaped loop of intestine was isolated. This loop had an intact blood, nerve and lymph supply and a readily accessible lumen. The continuity of the intestinal tract was restored by end-to-end anastomosis. The isolated loop was flushed daily with either physiological saline solution or phosphate-buffered saline solution (PBSS) warmed to 37°C.

Inoculation. Rotavirus for calf inoculation was cell culture supernatant fluid from rotavirus-inoculated bovine embryonic kidney cells. Calves 1 and 2 were inoculated orally at 8 and 9 days of age, respectively, with $2 \times 10^{6.5}$ 50% tissue culture infective doses ($TCID_{50}$) of virus. Calves 4 through 6 received $10^{6.5}$ $TCID_{50}$ of virus directly into the isolated intestinal loop. The inocula for calves 4 through 6 were treated with 100 μg of trypsin (1:250; Difco Laboratories, Detroit, Mich.) per ml of inoculum for 30 min at room temperature immediately before administration. This was done because the isolated loop did not contain trypsin and because rotavirus infectivity is enhanced by trypsin treatment (6, 9). Calf 3 was inoculated orally at 2 weeks of age with 2×10^4 $TCID_{50}$ of bovine rotavirus.

Collection and processing of intestinal washings. At selected intervals, the intestinal loop was flushed with 500 ml of PBSS. The first 25 to 30 ml of discharge fluid from calves 4 through 6 was collected for virus isolation and titration. The remainder of the fluid from all calves was collected for antibody assays.

Washings collected for antibody analysis were centrifuged at 3,000 × *g* for 30 min at 5°C. The supernatant fluid was placed into dialysis tubes and concentrated to 10 to 12 ml with polyethylene glycol (molecular weight, 20,000). This fluid was further concentrated with an ultrafiltration membrane filter (Amicon Corp., Lexington, Mass.). The resulting loop fluid concentrate was divided into aliquots and stored at −20°C. Immediately before antibody analyses, the protein concentration of the loop fluid was determined by the Bradford method (1), using bovine albumin as the standard. Protein concentrations were adjusted to 1 to 2 mg of protein per ml for all assays.

Neutralization tests. Neutralizing antibody titers to bovine rotavirus were determined on sera and intestinal washings with a microtransfer adaptation (5) of a previously described technique (3). Samples to be assayed were first inactivated by incubating in a water bath at 56°C for 30 min. Serial twofold dilutions of sera or intestinal washings were prepared, using microtiter transfer trays (Dynatech Laboratories, Inc., Alexandria, Va.). One hundred $TCID_{50}$ of bovine rotavirus in 0.025 ml of tissue culture medium was added to each well of the transfer tray, which was then incubated at 37°C for 60 min. The sample-virus mixtures in the transfer tray were transmitted to microtiter wells containing monolayers of MA-104 cells. Four wells were inoculated with each sample dilution. The plates were read by indirect immunofluorescence, using rabbit anti-bovine rotavirus serum and goat anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (Antibodies Inc., Davis, Calif.). Monolayers were stained and examined directly from the microtiter plates (33). Any well containing 10 or more fluorescent cells was read as positive; i.e., virus had not been neutralized. The titer was recorded as the reciprocal of the highest dilution of sample which neutralized virus in at least three of four wells. A fourfold or greater change in titer was significant. A known positive serum and virus back-titrations were included in all tests.

Immunofluorescent antibodies in intestinal washings. Monolayers of MA-104 cells grown in 96-well microtiter plates were washed, inoculated with 300 $TCID_{50}$ of bovine rotavirus, and incubated at 37°C. One day later, the monolayers were fixed with acetone–95% ethanol (6:4, vol/vol), air dried, and stored at 5°C. Plates were always used within 2 weeks.

Serial twofold dilutions of intestinal washings were prepared in PBSS. The fixed monolayers were washed with PBSS, covered with the appropriate dilution of intestinal washings, and incubated at 37°C for 30 min. Two wells were inoculated with each dilution. The monolayers were washed in PBSS, covered with fluorescein-conjugated goat antiserum to bovine immunoglobulin (Antibodies, Inc.), and again incubated at 37°C for 30 min. Finally, each well was rinsed twice with PBSS and once with distilled water. Plates were inverted and examined by epifluorescence. A known positive serum was used as a positive control. Monolayers not inoculated with virus served as negative controls. The titer was recorded as the reciprocal of the highest dilution of sample in which both inoculated wells contained clearly fluorescent cells.

Virus isolation and titration. Loop washings from calves 4 and 5 were centrifuged at 1,500 × *g* for 30 min at 5°C. The supernatant fluid was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). The filtrate was incubated with 100 μg of trypsin per ml for 30 min at 37°C. Tenfold dilutions were prepared in tissue culture medium containing 10 μg of gentamicin and 2.5 μg of amphotericin B per ml. Viral titration was completed as described previously (33).

Loop washings from calf 6 were divided into two equal portions. One portion was processed exactly as described above. The other portion was sonicated and then centrifuged at 1,500 × *g* for 30 min at 5°C. Antibiotics were added to the supernatant fluid, which was incubated with trypsin and titrated as described above.

Rotavirus antibody-producing cell count. Complete transverse sections of small intestine adjacent to the anastomosis site were collected at surgery. These tissues were processed for rotavirus antibody-producing cell detection. At approximately weekly intervals after virus inoculation, intestinal loop biopsies were obtained with a Rubin biopsy capsule and processed in the same manner. Four weeks after virus inoculation, the calf was anesthetized with pentobarbital, and transverse sections of duodenum, anastomosis, ileum, and isolated loop were removed for specific antibody-containing cell counts.

Rotavirus antibody-producing cells were detected by using alcohol-fixed, paraffin-embedded tissue blocks and an immunofluorescence sandwich technique reported previously (33). The immunofluorescence sandwich consisted of tissue sections containing

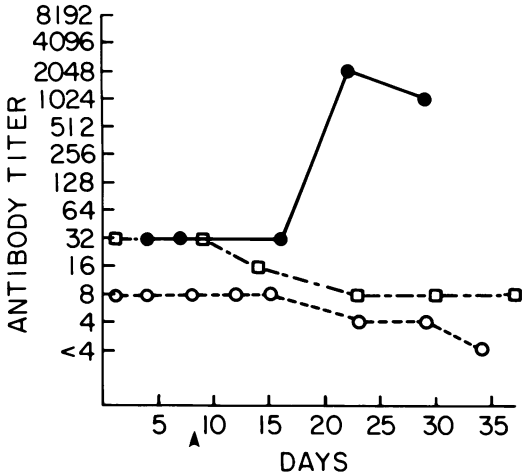

FIG. 1. Titers of neutralizing antibody in sera from calves 2 (□), 5 (○), and 6 (●). ▲, Virus inoculation.

rotavirus antibody-producing cells, a suspension of bovine rotavirus, and anti-bovine rotavirus serum conjugated to rhodamine. Cells producing antibody to rotavirus are stained red (rhodamine) by this technique. Control sections were treated in an identical manner; however, the rotavirus suspension was replaced by a noninfected cell suspension prepared similarly to the virus suspension. Tests for specificity consisted of blocking the fluorescent reaction with an unconjugated anti-bovine rotavirus serum and adsorption of the conjugate before use with a suspension of rotavirus.

Five histological sections from each intestinal sample were examined. Three sections served as principals, and two served as controls. The average number of rhodamine-stained cells in control sections was subtracted from the average number of stained cells in principal sections to determine net cell counts.

Double staining. The class or subclass of immunoglobulin in rotavirus antibody-containing cells was determined by using a double-staining procedure similar to that described previously (33). Either fluorescein-conjugated rabbit anti-bovine IgG1, IgG2, IgM, or IgA (Miles Laboratories, Inc., Research Products Div., Elkhart, Ind.) was used as the second fluorochrome-labeled serum. Only tissues containing 10 or more rotavirus antibody-producing cells were analyzed in this fashion.

Anti-bovine immunoglobulin conjugates were checked for heavy-chain specificity by blocking with the appropriate unconjugated antisera (Miles Laboratories). The anti-IgA and anti-IgM conjugates appeared to be specific. The anti-IgG1 and anti-IgG2 conjugates appeared to exhibit no cross-reactivity with respect to the anti-IgA and anti-IgM conjugates. However, it was never satisfactorily confirmed that the anti-IgG1 and anti-IgG2 conjugates were specific with respect to each other.

Double-stained sections were first examined with the rhodamine filter set to identify rotavirus antibody-containing cells. Once a rhodamine-stained cell was found, the filters were switched to the fluorescein filters to determine whether the cell also stained for the particular bovine immunoglobulin being sought at that time. Cells staining with the rhodamine conjugate only and with both the rhodamine and fluorescein conjugates were counted. If a tissue contained more than 100 rhodamine-stained cells, only 100 in each of three sections were counted. If a section contained fewer than 100 rhodamine-stained cells, all of the cells in the sections were counted. By totaling the number of double-stained and rhodamine-stained cells, the percentage of rotavirus antibody-containing cells producing IgG1, IgG2, IgM, or IgA could be determined.

Fluorescence microscopy. Microtiter plates and tissue sections were examined with a photomicroscope fitted with an epifluorescence condenser. The light source was a 50-W, high-pressure HB mercury lamp. A BG-38 red attenuation filter was used at all times. To determine whether a cell was stained by fluorescein conjugate, a 450–490 exciter filter, an FT 510 beam splitter, and an LP 520 barrier filter were used. To determine whether a cell was stained by the rhodamine conjugate, a BP546 exciter filter, an FT 580 beam splitter, and an LP 590 barrier filter were used. Photographs were taken on Kodak Ektachrome 160 tungsten film.

RESULTS

Significant changes in serum neutralizing antibody titers were observed only in calves 2, 5, and 6 (Fig. 1). Titers in calves 2 and 5 decreased from 32 to 8 and from 8 to <4, respectively, during the course of the study. Neutralization titers in calf 6 increased from 32 to a peak of 2,048 at 14 days after virus inoculation.

Neutralizing antibodies were detected in the intestinal washings from calf 6 only. Titers in this calf increased from <4 to 8. The rise in intestinal antibody titer coincided with the increase in serum neutralization titer.

Rotavirus antibodies were not detected by indirect immunofluorescence in the intestinal washings from any of the calves.

Attempts to isolate rotavirus from the intestinal washings of loop-inoculated calves 4 through 6 every 24 h for 5 to 10 days after inoculation were unsuccessful.

The rotavirus antibody-producing cells were primarily located in the lamina propria about intestinal crypts (Fig. 2), although occasionally they were found in the lamina propria of villous cores. In sections containing numerous fluorescent cells, the apices of crypt epithelial cells were brightly fluorescent (Fig. 3).

The amount and intensity of fluorescence were reduced by blocking the fluorescent reaction with an unconjugated antiserum to bovine rotavirus and by adsorbing the rhodamine conjugate with bovine rotavirus before use. Fluorescent cells were not observed when the rotavirus suspension was replaced with an uninfected cell suspension (control sections).

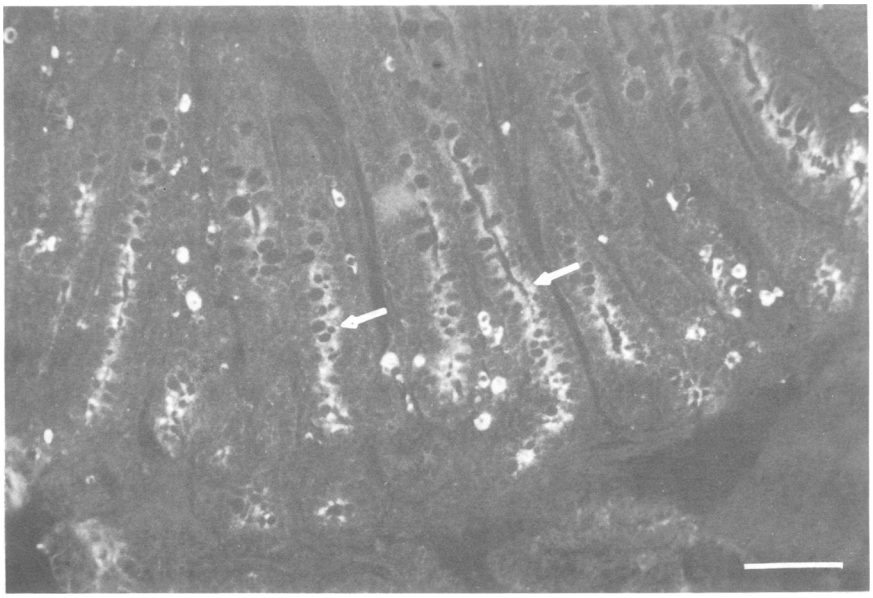

FIG. 2. Rotavirus antibody-producing cells scattered in the duodenal lamina propria in calf 1. Note the crypt fluorescence (arrows). Bar, 100 μm.

Rotavirus antibody-producing cells were not found in the weekly intestinal loop biopsies from any of the calves.

Counts of rotavirus antibody-producing cells in intestinal tissues collected at surgery and at the termination of each calf study are shown in Table 1. Rotavirus antibody-producing cells were found in all calves except calf 6; however, the counts varied widely among individual animals. The duodenal mucosa contained the major portion of the rotavirus antibody-producing cells.

Tissues containing 10 or more rotavirus antibody-producing cells were double stained to determine the class or subclass of immunoglobulin produced. Only duodenal sections from

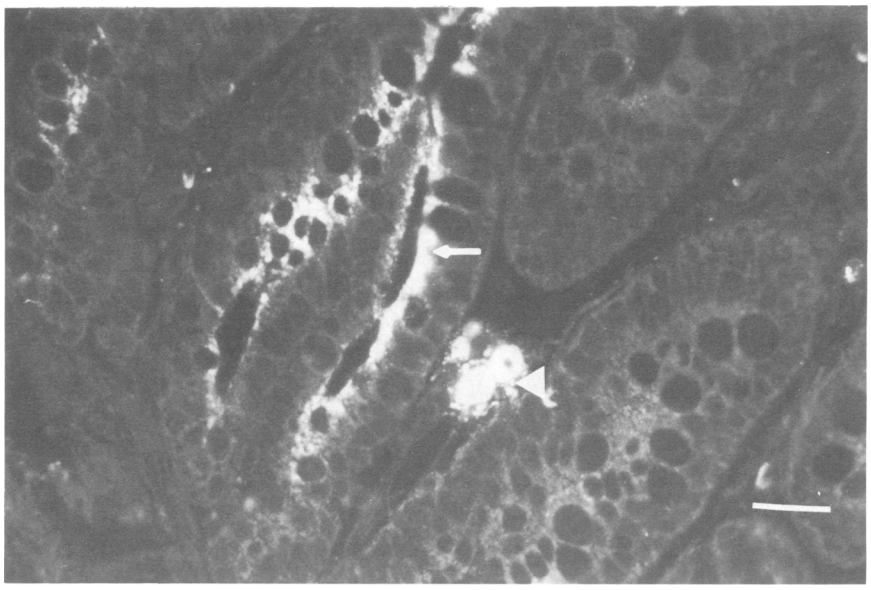

FIG. 3. Two rotavirus antibody-producing cells (arrowhead) in the duodenal mucosa of calf 3 surrounded by granular, fluorescent material. Note the bright crypt fluorescence (arrows). Bar, 20 μm.

TABLE 1. Rotavirus antibody-producing cells in intestinal mucosa

Calf no.	Pre-inoculation anastamosis site	Mean antibody titer ISD post-inoculation in[a]:			
		Duodenum	Anastamosis site	Ileum	Loop
1	0	>500	2.7 ± 0.6	1.3 ± 0.6	0.3 ± 0.6
2	0	31 ± 4.6	0.3 ± 0.6	2 ± 1.7	0
3	2 ± 1	277 ± 21.6	5.3 ± 2.5	0.3 ± 0.6	1.3 ± 0.6
4	0	65.7 ± 6.0	0	0	0
5	0	4 ± 1.7	1.3 ± 0.6	0	0
6	0	0	0	0	0

[a] Numbers represent the mean of three counts ± standard deviation.

calves 1 through 4 were analyzed in this fashion (Table 2).

Rotavirus antibody-producing cells contained primarily immunoglobulin of the IgA class (Fig. 4). IgA cells accounted for 71.8 to 94.3% of the cells producing rotavirus antibody. IgG1, IgG2, and IgM cells were also observed; however, in only one instance did the cells in any of these classes account for more than 10% of the cells containing antibody to rotavirus. Rotavirus antibody-producing cells containing IgA or IgM were located in the lamina propria adjacent to the intestinal crypts. Cells producing rotavirus antibody of the IgG1 or IgG2 subclass were often found in the villous cores. It was not possible to determine the class of rotavirus antibody in crypt epithelial cells since immunoglobulin in this location was stained by both the anti-IgA and the anti-IgM conjugates.

DISCUSSION

The specificity of the assay to detect rotavirus antibody-producing cells was tested by (i) blocking the virus-anti-rotavirus conjugate interaction with an unconjugated antiserum to bovine rotavirus, (ii) adsorbing the anti-rotavirus conjugate with bovine rotavirus before use, and (iii) replacing the rotavirus suspension with a noninfected cell suspension. Fluorescence was reduced in the first two tests and absent in the third. Thus, the system to detect rotavirus antibody-producing cells, which was developed in rabbits, was also specific in neonatal calves (33).

The major portion of the intestinal rotavirus antibody-producing cells contained antibody of the IgA class, indicating that the neonatal calves responded to rotavirus inoculation with a local IgA response. This is significant, since previous studies have shown that antibody must be present in the intestinal lumen to be effective in protection against rotavirus (29, 30). IgA is stable in the intestinal milieu, owing to its resistance to degradation by intestinal enzymes (10).

The rotavirus antibody in the crypt epithelium could be IgA, or IgM or both, since the crypt epithelium stained with both anti-IgA and anti-IgM conjugates. This was expected since both immunoglobulins share a common secretory pathway (2, 25, 26). The finding that the major proportion of rotavirus antibody-producing cells in the lamina propria contained IgA implies that the rotavirus antibody in the crypt epithelium was also IgA. This finding is consistent with the demonstration that rotavirus antibody activity in the feces of rotavirus-infected piglets is essentially due to IgA (7).

Nearly all of the rotavirus antibody-producing cells were found in the duodenal mucosa. Several investigators have suggested that intraluminal antigen plays a significant role in determining the distribution of antibody-producing cells in the intestinal lamina propria (11, 12, 22, 23). If antigen plays the major role in antibody-producing cell localization, the observation of rotavirus antibody-producing cells in the duodenum of loop-inoculated calves 4 and 5 can be explained if infection from the inoculum was spread to other parts of the gut either orally or systemically or if the calves received a natural infection, perhaps at the time of colostrum feeding, which resulted in immune response but not disease. Infection of the intestinal tract was documented in calf 4 by finding rotavirus-infected epithelial cells in fecal smears (data not shown.)

All six calves had serum neutralizing antibodies to rotavirus before virus inoculation. These antibodies most likely represent passive transfer of immunoglobulin from dam to calf via ingested

TABLE 2. Rotavirus antibody-producing cells: immunoglobulin classes and subclasses

Calf no.	% Cells belonging to:			
	Subclass		Class	
	IgG1	IgG2	IgM	IgA
1	2.0	0	2.0	93.0
2	8.2	13.7	4.3	71.8
3	2.7	4.3	.3	90.3
4	3.2	0	5.9	94.3
Average	4.0	4.5	3.1	87.4

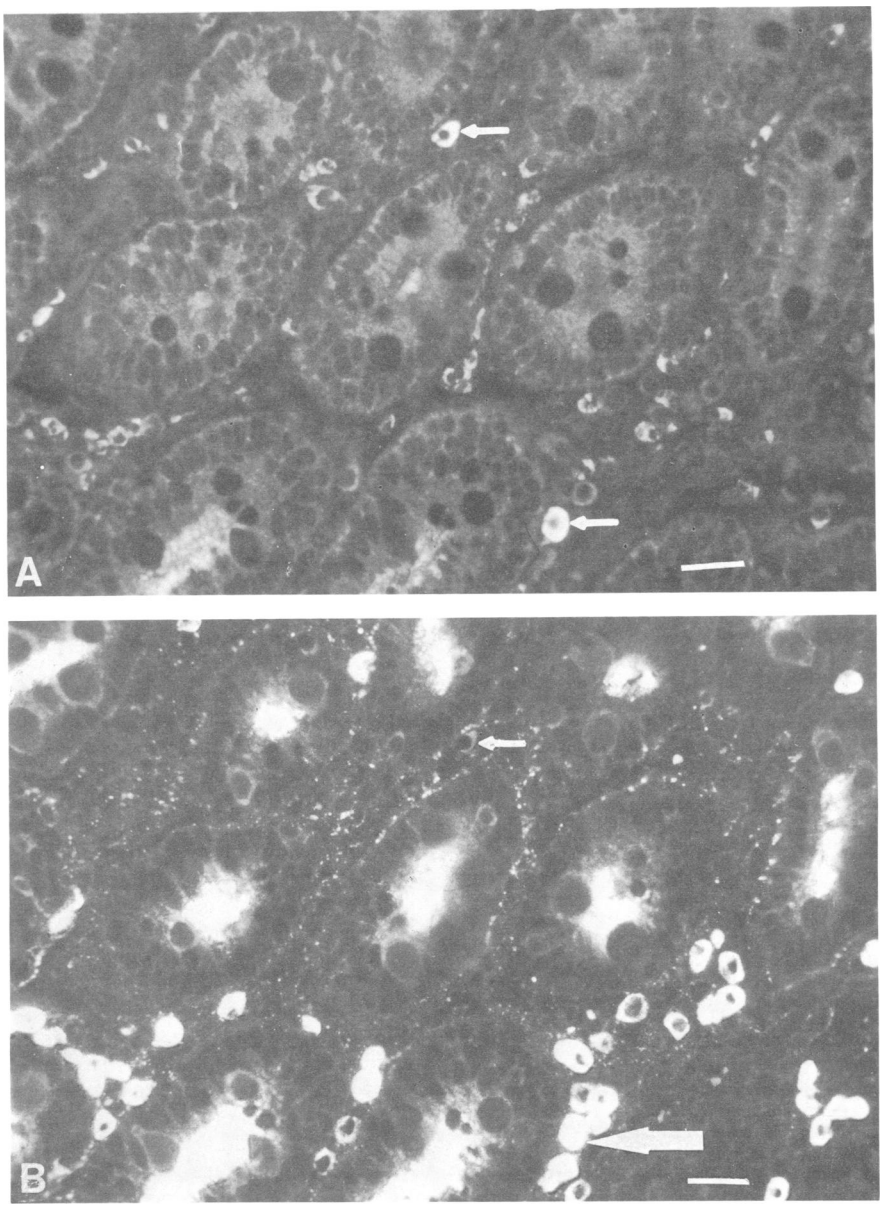

FIG. 4. Double staining of a duodenal section from calf 3. (A) Two rotavirus antibody-producing cells are present in the lamina propria (arrows). The dimly fluorescent cells scattered in the lamina propria are probably eosinophils. (B) One of the rotavirus antibody-producing cells (thick arrow) stained with fluorescein-conjugated anti-bovine IgA; the other did not (thin arrow). Bar, 25 μm.

colostrum. Bovine colostral immunoglobulin is primarily derived from the serum (4). Since a majority of cattle have serum antibodies to rotavirus (28), it follows that colostrum from a majority of cows would contain rotavirus antibodies. The decline in serum neutralizing antibody titers in calves 2 and 5 is compatible with catabolism of passively transferred immunoglobulin (14, 24).

Calf 6 was the only calf to respond serologically to rotavirus inoculation. Several intestinal pinch biopsies were obtained from this calf within 24 h after virus inoculation of the isolated intestinal loop; therefore, it is possible that viral particles entered the systemic circulation via the biopsy wounds and stimulated a systemic response in the absence of systemic viral replication. Despite numerous studies, no one has

reported systemic rotavirus infection without intestinal infection, and reports of systemic invasion of rotavirus are rare (18).

Neutralizing antibodies to rotavirus were found in the intestinal washings from calf 6 only. The antibodies, which first appeared 14 days after virus inoculation, could be either local or systemic in origin. The lack of rotavirus antibody-producing cells in the mucosa of the intestinal loop suggests that the antibody was derived from the systemic circulation.

Although neutralizing antibodies were detected in intestinal washings from calf 6, antibodies were not found in these washings by indirect immunofluorescence. Comparison of rotavirus neutralizing and fluorescent-antibody titers in sera, using assays similar to those used in the present study, has shown that fluorescent-antibody titers are consistently two- to fourfold lower than neutralizing titers (39). This would, of course, vary with the type of immunoglobulin present and the antigen(s) recognized by the antisera. An alternate explanation is that different antibody assay systems detect antibody to different viral proteins. This has been well documented with rotaviruses (13, 39). Exactly which viral proteins are important in the different assay systems is a question which deserves further investigation.

Rotavirus replication was studied in loop-inoculated calves only. Attempts to reisolate inoculated virus were unsuccessful, indicating that viral replication had not occurred. Several factors may account for the failure of rotavirus replication, including antibody in the intestinal loop, non-immunoglobulin virus inactivators, age of the calf, attenuated virus, and lack of trypsin in the isolated loop. Rotaviral replication may have been influenced by interaction among these factors. Exactly how the attenuated virus differs from the wild-type virus and how these differences are related to other factors are important questions. Unfortunately, too little is known about the behavior of the attenuated virus to provide adequate answers.

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