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Development of Resistance of Enterocytes to Rotavirus in Neonatal, Agammaglobulinemic Piglets†

CYNTHIA G. KIRSTEIN,‡ DEBRA A. CLARE, AND JAMES G. LECCE*

Grinnells Animal Health Laboratory, Department of Animal Science and Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7626

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The first part of this report describes the development of a technique for evaluating the growth of rotavirus under controlled conditions that approximate a natural infection. A standard dose of rotavirus (~10^9 viral particles) was injected into ligated segments in the small intestine of newborn, agammaglobulinemic, colostrum-deprived piglets. After various periods postinoculation, the segments were retrieved and the enterocytes were evaluated for the presence of rotaviral antigens by immunofluorescence and rotaviral particles by transmission electron microscopy. Peak immunofluorescence in enterocytes was detected at 8 h postinoculation in the upper and middle jejunum and ileum. Transmission electron microscopy at this time revealed fully formed virions which were not seen in sections examined before this 8-h period. The second part of our study describes the use of ligated segments in determining the susceptibility to rotavirus of enterocytes in piglets ranging in age from newborn to 2 weeks. By the time piglets were 2 days old, enterocytes in the upper half of the small intestines appeared to be resistant to rotavirus, whereas those in the lower half seemed partially resistant. Between 4 and 8 days of age, enterocytes in the lower half also became resistant. Resistance paralleled the loss in capacity of piglets to transport macromolecules through enterocytes and was not correlated with the loss in capacity to internalize macromolecules.

Previously, we reported that colostrum-deprived piglets expressed an age-dependent resistance to rotaviral infection (9, 19). Piglets infected with rotavirus when less than 1 day old experienced severe diarrhea and usually died, whereas peers infected at 10 to 12 days of age experienced a less severe diarrhea and no deaths. Since these piglets were agammaglobulinemic at birth (12, 20) and were reared in isolation, the age-dependent resistance did not result from passively or actively acquired immunity to rotavirus.

The epithelium of the small intestines of a neonatal piglet is in a state of flux regarding its ability to internalize and transport macromolecules. For example, in the first 2 days postpartum, macromolecules are nonselectively internalized (uptake) into enterocytes by pinocytosis and then transported into the blood (7, 12, 21, 22). For the next 2 to 3 weeks, macromolecules are still internalized into enterocytes but are not transported into the blood (14). During this 2- to 3-week period, cessation of uptake (closure) occurs beginning in the upper part of the small intestines at about 2 days postpartum and then proceeds towards the ileum, ending by 21 days postpartum (14).

To explain age-dependent resistance to rotavirus, we postulate that the mature, nonpinocytosing enterocytes are more resistant to rotaviral infection than immature enterocytes which are actively pinocytosing. However, before testing this hypothesis, a technique for evaluating the capacity of enterocytes to sustain a rotaviral infection was needed.

Such an evaluation would be difficult in piglets inoculated per os since the dosage of the virus would be diluted and possibly moderated by varying amounts of digestive secretions that contain inhibitors or enhancers of viral growth or both (3, 4, 11, 24). Also, the dosage of virus reaching the enterocytes would vary with respect to the location of the enterocytes in the small intestines. Enterocytes in the upper gut would receive proportionately more of the infecting dose, whereas enterocytes in the lower gut would receive both the infection dosage and progeny virus generated by enterocytes in the upper gut, leading to asynchronous growth.

Ideally, a system for evaluating the capacity of enterocytes to support rotaviral replication should maintain the physiological integrity of the gut, i.e., closely mimic natural conditions and not be complicated by such factors as the quality and quantity of digestive secretions, the location of the enterocytes within the gut, asynchrony of viral growth, or the immune status of the gut.

In the first part of this paper, we report on the development of a system for evaluating the growth of rotavirus under controlled conditions that approximate a natural infection. In this system, a standard dose of rotavirus was injected into ligated segments in the small intestines of agammaglobulinemic neonatal pigs. To determine an optimal incubation time, the segments were retrieved at various periods postinoculation (p.i.) and the enterocytes were evaluated for the presence of rotaviral antigens by immunofluorescence and rotaviral particles by transmission electron microscopy. From this study, we learned that 8 h p.i. was optimum for the detection of rotaviral antigens and particles.

The second part of this study describes the use of the ligated segment technique to determine at what age piglet enterocytes become resistant to rotaviral infection and whether there was a changing pattern of resistance in different areas of the small intestines as the piglet aged. From this study, we learned that resistance was more coincident with the cessation of transport of macromoelcules into the blood and that resistance did not parallel a loss in the ability of the enterocytes to internalize macromolecules (closure) (14).

* Corresponding author.

† Paper no. 9763 of the journal series of the North Carolina Research Service, Raleigh, NC 27695-7626.

‡ Present address: Burroughs Wellcome Research Institute, Research Triangle Park, NC 27709.

MATERIALS AND METHODS

Experimental animals. Piglets were farrowed from crossbred sows that were maintained in a closed herd at North Carolina State University.

Sixteen newborn, 17 1-week-, and 13 2-week-old colostrum-deprived piglets were used in experiments designed to determine the optimal incubation time for ligated segments to express rotaviral antigens.

Four newborns, four 2-day-, four 4-day-, three 8-day-, and four 2-week-old colostrum-deprived piglets were used to investigate the susceptibility of various areas of the small intestines to rotaviral infectivity as it relates to the age of the piglet.

One newborn and four 2-day-old, colostrum-deprived piglets were used to determine the capacity of enterocytes to internalize fluorescent immunoglobulin G (IgG).

All newborn piglets were denied food and used within 6 h of birth. Older piglets were denied food 12 h before surgery.

Piglets were farrowed in the Intensive Care Farrowing Facility (18). Each pig was caught at birth and placed in a fumigated, isolated nursery containing an automated feeding device (Autosow; DMF, Raleigh, N.C. (9, 13, 15). Since the piglets did not nurse, they were agammaglobulinemic (12, 20).

Piglets, other than those used within 6 h of birth, were individually caged and fed hourly a liquid diet that was similar to sow's milk, i.e., a dry weight of 20%, 32% of which is protein, 22% of which is fat, 42% of which is carbohydrate, and 8% of which is minerals (17). The volume fed was 12.5 ml/h per kg of weight of the piglet.

Viral inoculum. A pool of rotavirus was obtained by combining 30 samples of rotavirus-positive diarrhetic fluid obtained within a 10-day period from about 40 piglets with weanling diarrhea. These pigs had been weaned at 14 days of age from the Intensive Care Farrowing Facility to one of our isolation rooms. The pool was centrifuged at 7,970 × *g* for 40 min at 4°C to remove bacteria and debris. This pool was judged by electron microscopy to have ~10^9 rotaviral particles per ml, 40% of which were smooth particles (M. W. King and J. G. Lecce, Proc. Southeast Electron Microsc. Soc., 1980, abstr., p. 11). No other viruses were visualized. RNA electropherograms were done for this pool and for pools made before and after this pool. All were similar and did not seem to be mixtures of electropherotypes (M. W. King and J. G. Lecce, unpublished data). Samples of this pool were frozen at −20°C.

(i) Evaluating growth of rotavirus. Optimal incubation time. Piglets were anesthetized with methoxyfluorane (Metofane; Pitman-Moore, Inc., Washington Crossing, N.J.). A 5-cm midline incision was made in the abdomen, through which the small intestine was exteriorized. Two ligated segments 3 cm long were tied in the upper jejunum, middle jejunum, and ileum for a total of six segments. Upper and middle jejunum and ileum corresponded to the beginning of zone 2, the beginning of zone 3, and the lower half of zone 4, respectively (see below). Approximately 1 ml of the rotavirus pool was injected into each of the segments. A control segment, located between the middle and distal segments, was injected with 1 ml of Hanks balanced salt solution. The exposed small intestine was placed back into the peritoneal cavity, and the incision closed.

After the virus incubated within the gut segments for 2, 5, 8, 12, or 24 h p.i., the piglets were anesthetized with sodium pentobarbital and exsanguinated. Ligated segments were retrieved from the intestine, everted, washed, and sliced into four rings. Two rings were fixed overnight at 4°C in 95% ethanol for indirect immunofluorescent microscopy, and the contiguous two rings were fixed in 4% (vol/vol) glutaraldehyde in cacodylate buffer (pH 7.2, 0.1 M) at 4°C for transmission electron microscopy. Blood collected at this time was tested for antibodies to rotavirus by indirect immunofluorescence, using known rotavirus-positive sections of piglet small intestine.

Indirect immunofluorescent microscopy. To test for the presence of rotaviral antigens, the ethanol-fixed gut rings were dehydrated and infiltrated with paraffin under vacuum, and sections were cut at 5 μm (29). Sections were stained by indirect immunofluorescence with rabbit anti-porcine rotavirus (made in our laboratory) and fluorescein-conjugated goat anti-rabbit gamma globulin (Cappel Laboratories, Cochranville, Pa.).

Fluorescence score. Fluorescence within the columnar epithelium was scored by scanning each intestinal ring. Essentially, samples exhibited two different degrees of fluorescence: strong (5+), abundant, bright fluorescent enterocytes on most villi (see Fig. 1A); weak (0.5+), occasional, fluorescent enterocytes scattered about on the villi, similar to that depicted in Fig. 1B; and negative, no fluorescent enterocytes on the villi.

Transmission electron microscopy. Glutaraldehyde-fixed intestinal rings stored at 4°C were selected for further processing if the contiguous rings were positive by immunofluorescence. The tissue was then cut into 1-mm^3 blocks, fixed in 1% osmium tetroxide for 1 h at 25°C, dehydrated in increasing concentrations of ethanol, and added to propylene oxide before being embedded in Epon. Ultrathin sections were cut at 600Å with a diamond knife on a Porter-Blum MT2 ultramicrotome. Sections were then stained for 1 h with an aqueous solution of saturated uranyl nitrate and then saturated again with a solution of lead citrate for 2 min (27). Sections were examined at 80 kV with a Siemen's Elmiscope I transmission electron microscope.

(ii) Effect of age on susceptibility of different areas of small intestines to rotavirus. Four sampling zones. Piglets were anesthetized, and the small intestines were exteriorized as described above. The area between the upper jejunum and ileum was subdivided into four zones. Zone 1 represented an area 6 to 16% from the pylorus; zone 2 was 25 to 50% from the pylorus, zone 3 was 50 to 75% from the pylorus, and zone 4 was 75 to 100% from the pylorus.

In each zone, ligated segments 1 cm long and 1 cm apart were injected with 1 ml of the undiluted rotavirus pool and 1 ml each of 1:40, 1:160, and 1:640 dilutions of the pool in Hanks balanced salt solution. The gut was returned to the peritoneum, and 8 h later, the ligated segments were retrieved and enterocytes were evaluated as above by immunofluorescence for the presence of rotaviral antigens.

Fluorescent intensity (0.5+ to 5+). Since viral dilutions were used, it was possible to grade the degree of fluorescence by approximating the number of positive enterocytes present on the villi as follows: 0.5+, <10% of the enterocytes were positive; 1+, ~25% were positive; 2+, ~50%; 3+, ~75%; 4+, ~90%; 5+, >90% (Fig. 1A shows 5+, and 1B shows 0.5+).

Average fluorescent intensity for each dilution. To determine the susceptibility of a particular zone to rotavirus infection, the fluorescent intensity score for each dilution within a zone was summed and divided by the number of piglets used, yielding the average fluorescent intensity for each dilution (see Fig. 3).

Capacity of enterocytes to internalize macromolecules. Flu-

orescein-tagged porcine IgG prepared in our laboratory was used as a macromolecular marker to determine which zone in the small intestines still had pinocytosing enterocytes. By 2 days of age, piglets no longer transport macromolecules from their gut lumen to the blood, but enterocytes in the lower parts of the small intestines can still internalize macromolecules (14).

The small intestines of the piglets were exteriorized and divided into four zones as described above. In each zone, 1-cm-long ligated segments were injected with 20, 10, 5, and 2.5 mg respectively, of fluorescein-labeled porcine IgG. After 2 h the ligated segments were retrieved and processed as described above. The sections required no further staining with fluorescent reagents since fluorescein was attached to the IgG. Enterocytes within a specific zone were evaluated for the presence of the fluorescent marker (0.5+ to 5+). Average fluorescent intensity per milligram of IgG was calculated.

TABLE 1. Immunofluorescence of enterocytes in ligated segments injected with rotavirus in newborn[a] colostrum-deprived piglets

Incubation time (h p.i.)	No. of pigs	Upper jejunum			Middle jejunum			Ileum		
		No. of samples	% 0.5+[b]	% 5+[c]	No. of samples	% 0.5+	% 5+	No. of samples	% 0.5+	% 5+
2	3	12	8[d]	0	11	18	0	12	17	0
5	4	16	19	38	16	31	19	16	25	50
8	3	11	0	91	12	0	100	12	0	100
12	3	8	50	37	10	20	80	10	40	40
24	3	8	37	13	8	75	0	9	33	11

[a] In 1- and 2-week-old piglets no samples were 5+; about 50% were 0.5+ in upper and middle jejunum and ileum.
[b] Occasional fluorescent enterocytes scattered about on villi (weak).
[c] Abundant, brightly stained enterocytes on most villi (strong).
[d] % Samples.

RESULTS

Antibodies to rotavirus were not detected in the sera of colostrum-deprived animals prior to experimental infection.

(i) Evaluating growth of rotavirus. Optimal incubation time. Upper jejunum. In newborn piglets, peak fluorescence was observed 8 h after the ligated segments were inoculated with rotavirus. Ninety-one percent of the samples had a strong fluorescent reaction (5+) (Table 1, Fig. 1A). Fewer samples were strongly positive (Fig. 1B) at 5, 12, or 24 h p.i. (38, 37, and 13%, respectively).

Middle jejunum. The same trend observed in the upper jejunum was seen in the middle jejunum. Again, peak fluorescence occurred 8 h p.i. (100% 5+). At 5 and 12 h p.i., 19 and 80%, respectively, of the samples were 5+.

Ileum. As was the case with the upper and middle jejunum, peak fluorescence occurred at 8 h p.i. (100% 5+). After 5, 12, and 24 h p.i., 50, 40, and 11% of the samples, respectively, exhibited a 5+ score.

Unlike the newborn piglet, the strongly positive fluorescent reaction (5+) was rarely seen in the upper and middle jejunum or ileum of 1- and 2-week-old piglets. About 50% of the samples in the upper and middle jejunum and ileum exhibited fluorescent enterocytes scattered about the villi after 8 to 12 h of incubation (0.5+; Fig. 1B).

Transmission electron microscopy. In newborn pigs fully formed, mature virions were seen only in segments harvested at 8, 12, and 24 h p.i. in the upper and middle jejunum and ileum (Fig. 2). The arrow points to a swollen rough endoplasmic reticulum (RER) containing rotaviral particles in an infected enterocyte (Fig. 2A). This area, magnified further (inset B), revealed a convoluted vesicular membrane (double arrows) near the swollen RER that contained 75-nm particles. Often 36-nm virus-like particles were seen in the viroplasm (inset D, arrow). At the periphery of the viroplasm, 75-nm particles seemed to be budding into the RER (insets C and D, arrow heads). Also seen within the RER were 68-nm particles which lacked a distinct outer layer. These particles (inset C, double arrow) were joined together by kinky filaments (inset C, arrow).

Neither rotavirus nor any morphological alteration in enterocytes was observed at 2 or 5 h p.i. The morphology of control enterocytes injected with Hanks balanced salt solution also appeared normal.

No detectable rotaviral particles were observed in the intestinal epithelium of the 1- and 2-week-old piglets at any of the incubation times. Difficulty in finding positive cells was expected since so few enterocytes were infected according to the results from immunofluorescence.

(ii) Effect of age on susceptibility of different areas of small intestine to rotavirus. In the newborn piglet the upper 6 to 16% of the small intestine appeared to be resistant to rotaviral infection (Fig. 3); however, at a distance of about 25% from the pylorus, enterocytes were susceptible to rotavirus. The lower half of the small intestine seemed to be more susceptible to rotaviral infectivity than the area 25 to 50% from the pylorus.

By the time the piglet was 2 days old, the upper 50% of the small intestine was apparently resistant to rotaviral infection and there was a marked drop in the susceptibility to infection in the lower half of the gut. A similar pattern was seen in 4-day-old piglets. Results for 8- and 14-day-old piglets were essentially negative in that only an occasional, weakly fluorescent enterocyte was seen scattered throughout all areas of the small intestines (Fig. 1B).

Capacity of enterocytes to internalize macromolecules. In the newborn piglet fluorescent IgG was internalized to about the same extent by enterocytes in all zones (Fig. 4). Only enterocytes in the lower 75% of the small intestines were capable of internalizing fluorescent porcine IgG in the 2-day-old piglet. These results were similar to those reported previously (14).

DISCUSSION

Mammals express an age-dependent resistance to enteroviruses that is independent of the immune response (9, 19, 23, 28, 31, 32). For example, the small intestines of nursing mice are susceptible to infection by rotavirus and coxsackievirus, whereas weanling mice are resistant. As previously mentioned, piglets are much less affected by rotavirus when infected around 1 to 2 weeks of age as compared to an infection occurring within the first 2 days of life (19).

The absorption of macromolecules by the neonatal gut occurs in at least two phases (14). The first phase includes an initial uptake by pinocytosis of macromolecules into the enterocyte. During the second phase, macromolecules are then transported out of the enterocyte into the lamina propia. Mouse enterocytes lose both uptake and transport capacity at 17 days of age (6, 12). Piglet enterocytes lose transport capacity within the first 2 days of life, whereas internalization of macromolecules occurs for another 2

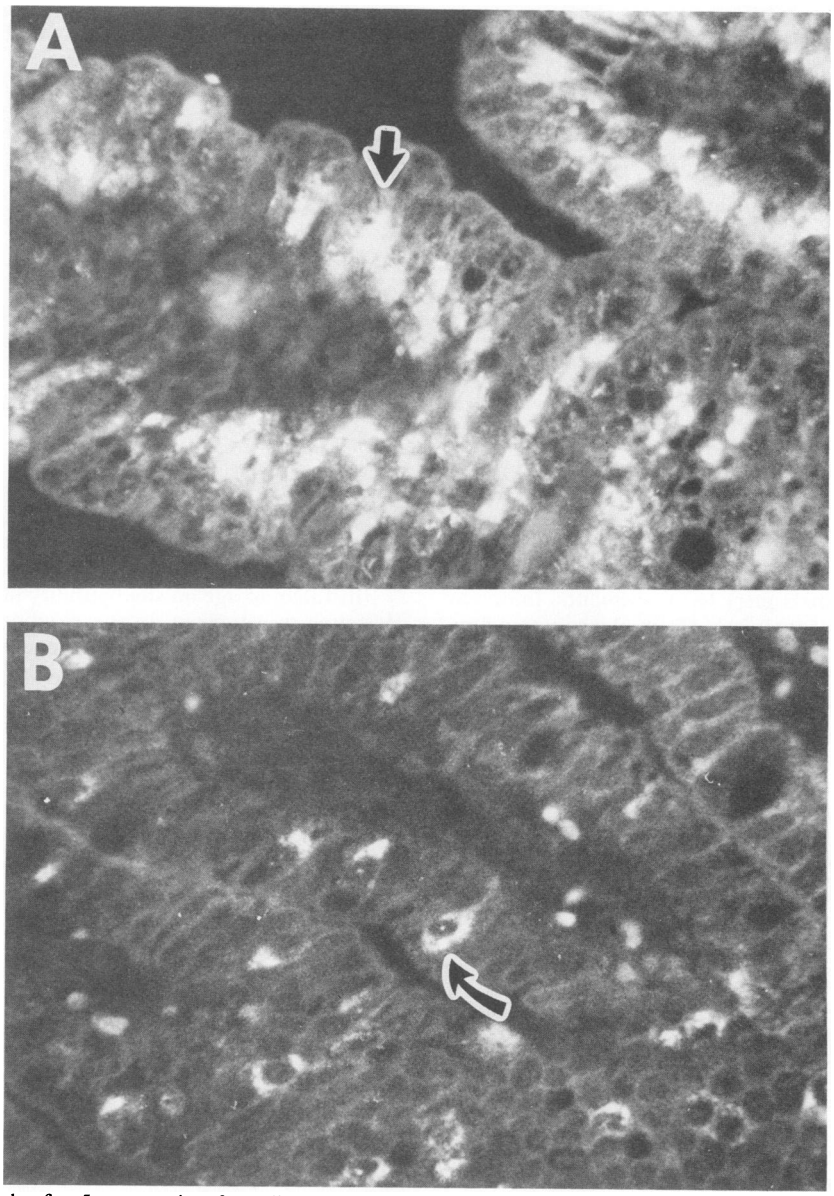

FIG. 1. Photomicrograph of a 5-μm section from ligated segments from the jejunum of a newborn piglet (50 to 75% from pylorus). Segments were injected with dilutions of rotavirus, and 8 h later segments were retrieved, processed, and stained for the presence of rotaviral antigens by indirect immunofluorescence. (A) Segment injected with undiluted rotavirus, 5+. Arrow points to villus with >90% fluorescent enterocytes. (B) Segment injected with rotavirus diluted 1:160, 0.5+. Arrow points to villus with <10% of fluorescent enterocytes. Magnification, ×735.

weeks in the lower 75% of the small intestines (Fig. 4; reference 14).

The intent of the work reported here was to determine whether enterocytes in older piglets were more resistant to rotaviral infection than enterocytes in younger piglets. If so, then does this diminished rotaviral infective capacity in enterocytes of piglets correlate with a loss in transport or with a cessation in capacity to internalize macromolecules (closure)?

To address these questions, we developed a system that could be used to evaluate the growth of rotavirus under controlled conditions that approximate a natural infection. In this system, a rotavirus inoculum was injected into ligated segments in the jejunum and ileum of agammaglobulinemic piglets. Replication of rotavirus in ligated segments appeared to be normal in that all of the structures that have been visualized in enterocytes by immunofluorescence and electron microscopy during natural infections and in tissue cultured cells were seen in the ligated segments infected with rotavirus (1, 2, 5, 8, 10, 25, 26, 30).

Using ligated segments, we learned that there was some resistance to rotavirus infection by the time piglets were 2 to 4 days old. Notably, enterocytes in the upper half of the small intestine were resistant and enterocytes in the lower half had diminished infective capacity (Fig. 3). Between 4 and 8 days of age, enterocytes in the lower half also became resistant. These results indicate that diminished infective capacity was more coincident with a loss in transport capacity than closure, since enterocytes in the lower 50% of the small intestines, which were resistant to rotavirus infection,

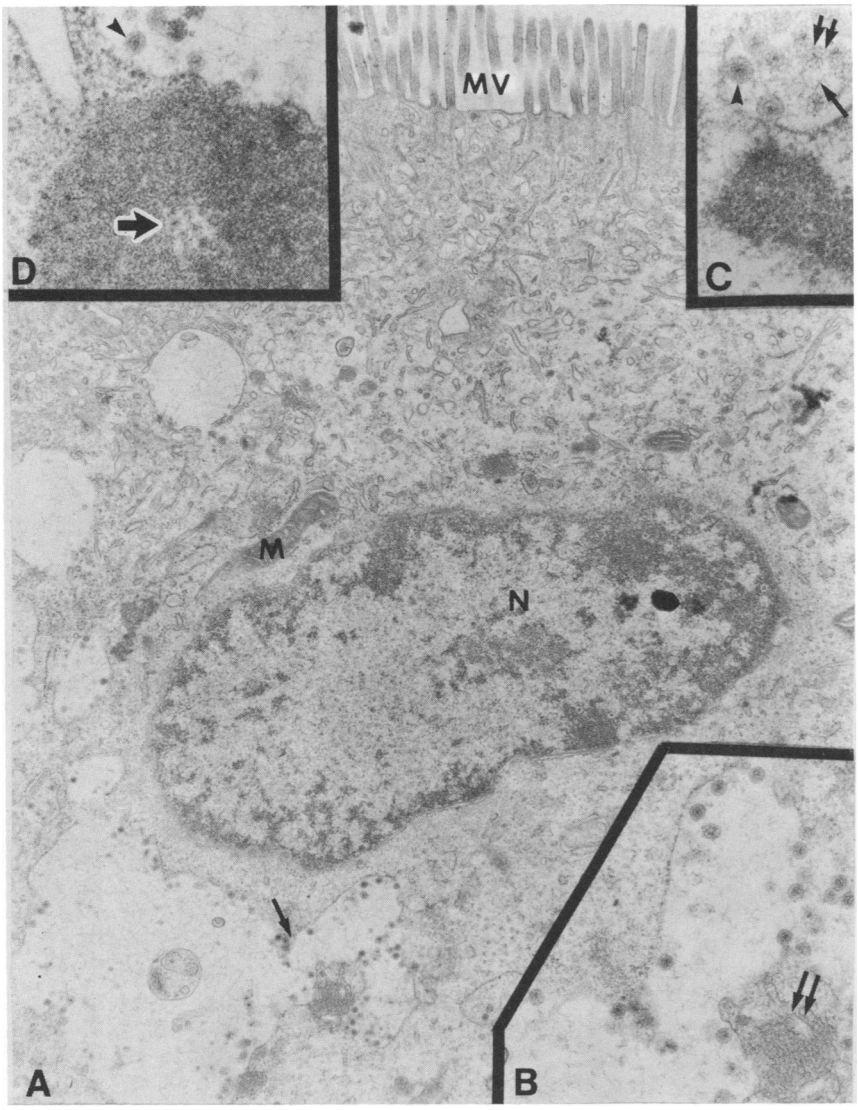

FIG. 2. Electron micrographs made from ligated segments from a newborn colostrum-deprived piglet at 12-h postinoculation with rotavirus. N, Nucleus; m, mitochondria; MV, microvilli. (A) Enterocyte with RER containing viral particles (arrow). Magnification, ×9,450. (B) Higher magnification of RER; convoluted membrane (double arrows) is adjacent to RER containing 75-nm particles. Magnification, ×22,050. (C) Particles (75-nm; arrowhead) with a distinct outer layer appear to bud into the RER from the adjacent granular viroplasm; 68-nm particles (double arrows) and kinky filaments (single arrow) are also shown. Magnification, ×29,295. (D) Granular viroplasm containing 36-nm particles (heavy arrow); viroplasm is adjacent to RER containing 75-nm particles (arrowhead). Magnification, ×31,500.

can continue to pinocytose macromolecules such as immunoglobulins for up to 3 weeks (Fig. 4; reference 14).

Perhaps the age-dependent resistance to rotavirus infection involves specific receptors for the virus. That is to say, there are abundant rotaviral receptors on the plasmallema of the actively pinocytosing enterocytes in piglets less than 2 days of age. As the piglet ages, and coincident with a loss in transport capacity, the number of receptors decreases throughout the small intestine. Even though enterocytes in the lower part of the small intestine are still capable of pinocytosing macromolecules, these enterocytes have a lower number of receptors and are not efficiently infected. It would follow then that pinocytosis per se does not lead to efficient infection. This hypothesis is supported by a report which suggests that enterocytes in mice are more efficiently infected when mice are 6 to 11 days old, at which time enterocytes possess the greatest number of rotaviral binding sites (28). Both susceptibility of enterocytes to infection by rotavirus and binding of rotavirus to enterocytes declines rapidly after this time (28). However, loss of transport capacity and closure (pinocytosing capacity) does not occur until mice are 17 days old (6, 12).

The stark difference between the susceptibility of enterocytes in newborn piglets versus that in 1- and 2-week-old piglets could convey the impression that piglets rapidly become resistant to rotaviral diarrhea as they age. Yet, this is not entirely so because rotavirus is an important etiological agent in the diarrhea experienced by 3- to 4-week-old piglets (16).

Highly resistant enterocytes on one hand and rotaviral weanling diarrhea on the other hand seems like a paradox. However, bear in mind, many factors are at play in the balance between infection and disease. Consider the case of piglets at weaning. One moment the piglets are content with

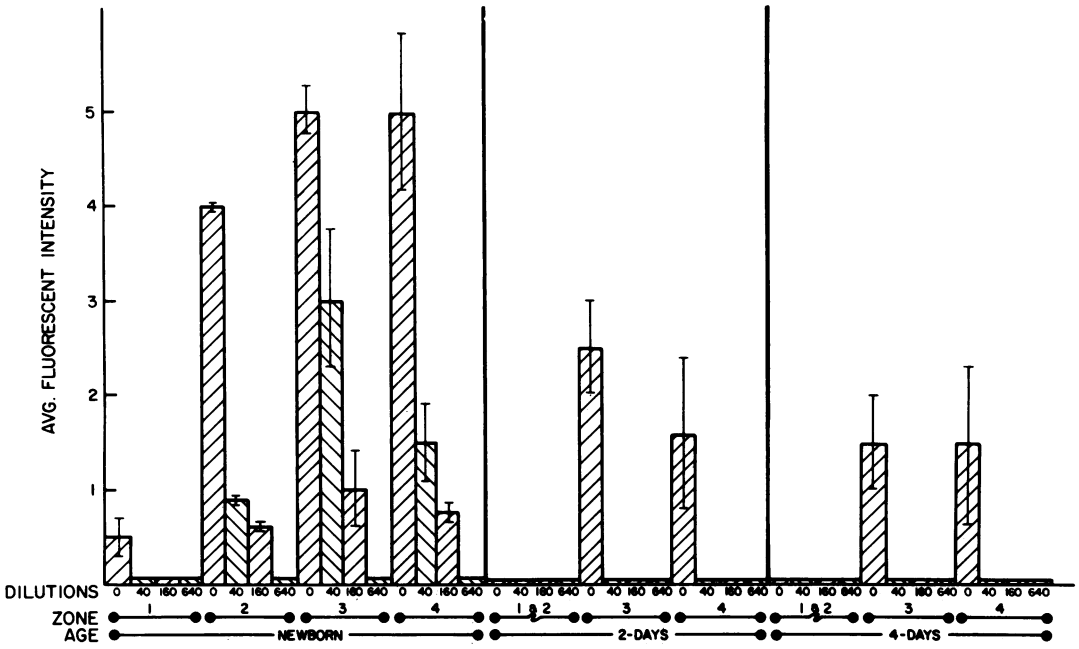

FIG. 3. Average fluorescent intensity per rotavirus dilution in four different zones in small intestines of piglets. Fluorescent intensity score for each dilution in a zone was summed and divided by the number of piglets. The small intestine was divided into four zones: zone 1, 6 to 16% from pylorus; zone 2, 25 to 50% from pylorus; zone 3, 50 to 75% from pylorus; and zone 4, 75 to 100% from pylorus. In each zone, ligated intestinal segments were injected with four different dilutions of rotavirus (undiluted, 1:40, 1:60, 1:640). Vertical lines are standard error.

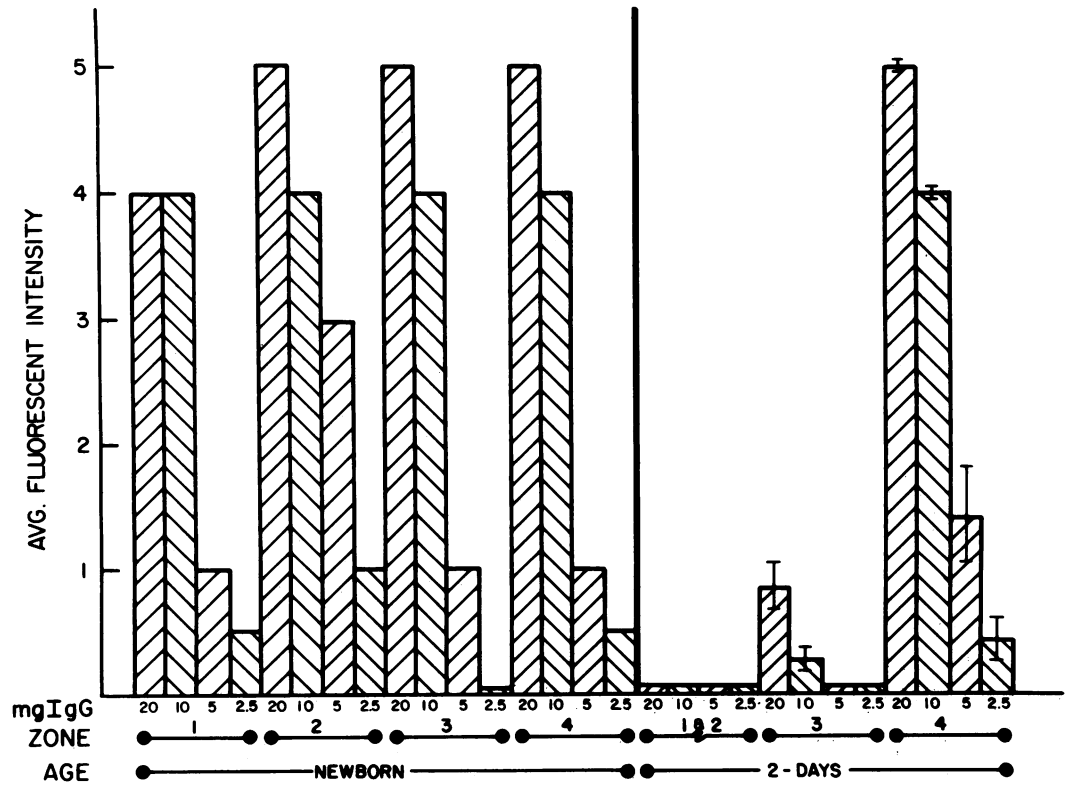

FIG. 4. Average fluorescent intensity per milligram of fluorescent IgG in four different zones in small intestines of piglets. Fluorescent intensity score for each milligram of fluorescent IgG in a zone was summed and divided by the number of piglets. Small intestine was divided into four zones as described in the legend to Fig. 3. In each zone, ligated intestinal segments were injected with four different amounts of fluorescent IgG (20, 10, 5, and 2.5 mg). Vertical lines are standard error.

their siblings and are being fed hourly a liquid diet containing antibodies to enteropathogens,-including rotavirus. The next moment these piglets are abruptly taken from their dam and the protective antibody bathing their gut. Next, the weaned piglets are moved into a building contaminated with rotavirus and other enteropathogens. Here, they are regrouped and presented with a dry diet.

Thus, at weaning piglets are suddenly confronted with a change in diet, a need to reestablish a new social order, a lack of humoral gut immunity, and constant contact with enteropathogens (both in the environment and in recycled feces). Most of these factors that can impinge on and influence the course of a natural infection are missing in the ligated segments, in which dietary changes, social realignment, and recycling of enteropathogens are not involved.

In summary, we reported previously in piglets infected per os with rotavirus (19) and reaffirm here in detail in ligated segments injected with rotavirus an age-dependent resistance to rotaviral infection that is not attributable to acquired active or passive immunity. Resistance of enterocytes to infection correlates with the cessation of transport of macromolecules and may be involved with the loss of rotaviral receptors.

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