Single and Mixed Infections of Neonatal Pigs with Rotaviruses and Enteroviruses: Clinical Signs and Microscopic Lesions

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ABSTRACT

Neonatal colostrum-deprived pigs were inoculated with cell-culture preparations of three rotaviruses and three enteroviruses, singly or in combination. The three enteroviruses established intestinal and systemic infection but did not induce diarrhea or intestinal lesions. The three rotaviruses produced severe enteric disease characterized by profuse watery diarrhea, dehydration and death. Villi were severely stunted. All three isolates were equally virulent. Inoculation with three different rotavirus-enterovirus combinations resulted in disease less severe than that produced by the rotaviruses alone. Intestinal lesions were less extensive and fewer pigs became moribund or died.

RÉSUMÉ

Cette expérience consistait à faire ingérer à des porcelets nouveau-nés et privés de colostrum, des préparations de cultures tissulaires de trois rotavirus et d'autant d'entérovirus, seuls ou en mélanges. Les trois entérovirus déclenchèrent une infection intestinale et systémique, sans toutefois provoquer de diarrhée ou de lésions intestinales. Les trois rotavirus provoquèrent une entérite sévère qui entraîna une diarrhée profuse, de la déshydratation et la mort. Les villosités intestinales subirent une atrophie marquée et les trois isolats affichèrent la même virulence. L'administration buccale de trois mélanges de rotavirus et d'entérovirus se traduisit par une maladie moins grave que celle qui suivit l'ingestion de seulement les rotavirus. Les lésions intestinales se révélèrent moins extensives et moins de porcelets devinrent moribonds ou succombèrent à l'infection.

INTRODUCTION

Rotaviral diarrhea is a common problem in swine-rearing operations (1). The disease has been reproduced by experimental inoculation, and the pathogenesis of diarrhea induced by this virus has been studied by numerous researchers (2-6). Variability in the severity of disease resulting from rotavirus infection is observed both in field situations and in laboratory studies (1,7,8). These observations suggest that additional factors may modify or ultimately determine the outcome of rotavirus infection. A number of factors have been proposed, including dose and virulence of virus, age of pigs at infection, level of immunity in affected pigs, diet, environmental stress, and concurrent infection with other infectious agents (1,7,9-14).

Enteroviruses also infect the intestinal tract of pigs and have been associated with diarrhea. However, experimental studies have not conclusively established these viruses as enteric pathogens (15-16). Diarrhea is not seen consistently and no intestinal lesions are found after experimental inoculation. Both rotaviruses and enteroviruses are ubiquitous in swine populations (1,15,16), and these viruses have been observed together in the feces of diarrheic pigs (3,8). However, the effect of concurrent infection has not been studied.

Diarrheic pigs infected with both rotavirus and enterovirus from four different herds were received at the Veterinary Medical Diagnostic Laboratory at the University of Missouri. Three of the enteroviruses and one of the rotaviruses recovered from these cases were selected for further study. In addition, two other rotaviruses recovered from field cases were studied. The purpose was to compare the relative virulence of these six isolates and to determine the effect of concurrent infection with rotavirus and enterovirus.

MATERIALS AND METHODS

VIRUSES

Three enteroviruses were isolated from the intestines of diarrheic pigs in swine testis (ST) cell monolayers and propagated in this cell line. The serotype of each isolate was determined by neutralization test against commercial typing sera. The following

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Published as Journal Series No. 10125. Contributions from the Missouri Agricultural Experiment Station.

Submitted July 16, 1987.

inocula were prepared from these isolates: 6167E (Type 7), eighth cell culture passage, 1 x $10^{7.8}$ $TCID_{50}$/mL; 6335 (Type 2), fourth passage, 1 x $10^{7.2}$ $TCID_{50}$/mL; 10841 (Type 3), 11th passage, 1 x $10^{8.4}$ $TCID_{50}$/mL (17).

Three rotaviruses were isolated and propagated in rhesus monkey kidney (MA-104) cell monolayers using pancreatin for pretreatment of virus and in cell culture maintenance medium. All three isolates were identified as Group A serotype 1 porcine rotaviruses. The following inocula were prepared from these isolates: 6167R, 13th cell culture passage, 1 x $10^{6.5}$ $TCID_{50}$/mL; 6418, sixth passage, 1 x $10^{6.5}$ $TCID_{50}$/mL; 10986, seventh passage, 1 x $10^{6.5}$ $TCID_{50}$/mL (17).

EXPERIMENTAL DESIGN

Fifty-four colostrum-deprived neonatal pigs were obtained by hysterectomy or by catching in sterile bags at farrowing. Pigs were placed immediately into individual cardboard isolators and fed homogenized, pasteurized cows' milk in metal trays every 6 h. At one day of age, pigs were inoculated orally with 1 mL of either rotavirus or enterovirus, or with 1 mL of both viruses. Control pigs were given uninoculated cell culture lysates. Nineteen pigs received rotavirus, 12 pigs received enterovirus, 15 pigs received rotavirus and enterovirus, and eight pigs served as controls. At 6 h intervals, before feeding, pigs were observed for appetite, condition, and fecal consistency.

NECROPSY

Pigs from each group were euthanized by electrocution at 24 h intervals. Pigs that became moribund between these intervals also were euthanized and sampled to prevent loss of tissue and experimental data to postmortem autolysis. All pigs were processed in a similar manner. The gross appearance of the intestines and the consistency of intestinal contents were noted. Immediately after euthanasia, 2-3 cm segments of small intestine were fixed in 10% buffered formalin for histopathological examination. A section of duodenum was taken just distal to the pylorus. A section of lower ileum was taken just proximal to the ileocecocolic junction. A section of upper ileum was taken 15 cm cranial to the lower section. Three sections of jejunum were taken at approximately equal intervals between the duodenal sample and the upper ileal sample. Samples of stomach, colon, mesenteric lymph node, lung, spleen, liver, kidney and brain also were taken for histopathological examination.

The following features were evaluated by histopathological examination for each level of small intestine: villous length, crypt depth, morphology of villous epithelium, cellularity of the lamina propria, and inflammation or necrosis. Relative villous length was determined according to the following criteria. The long, thin jejunal villi of control pigs were considered the standard of maximum length (4+) (Fig. 1A). Villi that were slightly shorter were assigned to the next lower category (3+) (Fig. 1B). Villi approximately one-half to one-third the normal length were regarded as moderately stunted (2+) (Fig. 1C). When only short, blunt stumps remained, villi were considered severely stunted (1+) (Fig. 1D). The lowest grade (0) was given to those sections in which villous structure was no longer present (Fig. 1E). Mean villous length scores for each level of intestine were calculated for each group of pigs. In the ileum, villi not over Peyer's patches were used for the score for those sections. Results were analyzed statistically using the pooled variance t-test. Histopathological evaluation was not conducted on intestines of pigs that died.

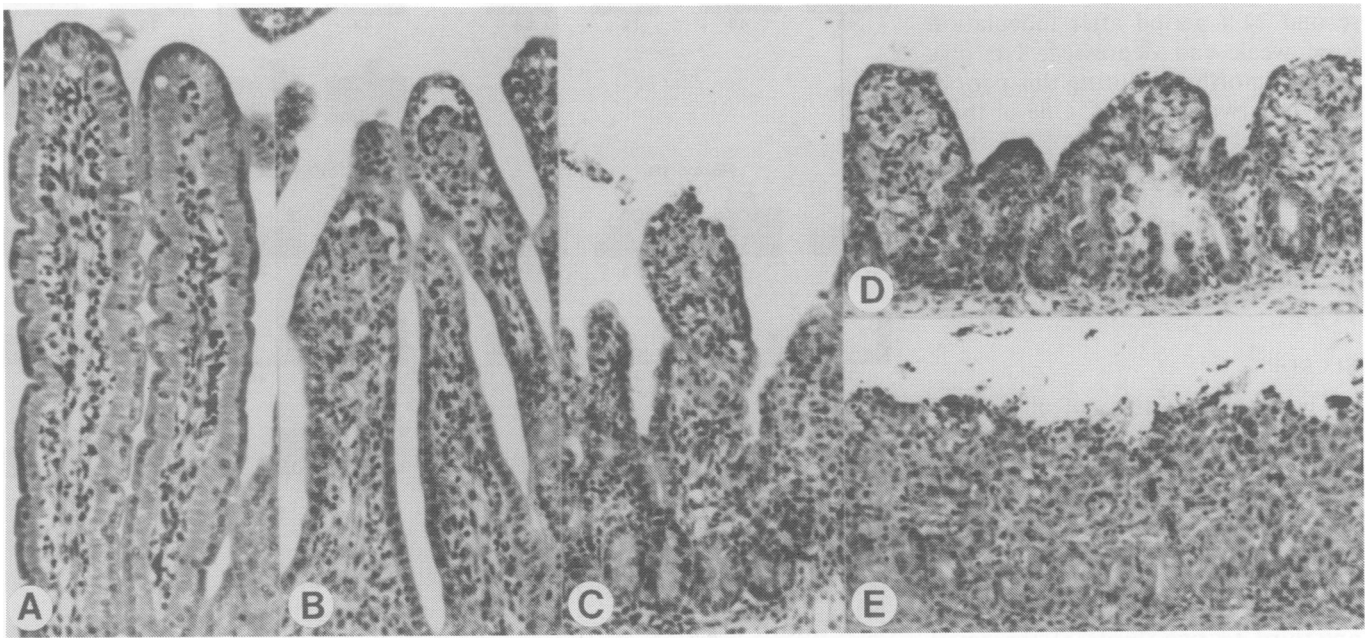

Fig. 1. Relative lengths of villi used as standards in evaluation of intestines from experimental pigs. Rating of villous length against these standards resulted in data presented in Fig. 2. Lengths were graded from 4+(A) to 0(E). H & E. X460.

RESULTS

CLINICAL SIGNS

Control pigs remained alert, hungry, and in good condition. Feces were bright yellow and firm or occasionally semisolid. No illness was observed in enterovirus-inoculated pigs. Fecal consistency was similar to that of control pigs.

Sixteen of 19 pigs inoculated with rotavirus developed watery diarrhea beginning 18 to 42 h (mean 22.5 h) PI. Feces remained fluid until the pigs were euthanized. There was impaction of the apex of the spiral colon in the three pigs that did not develop diarrhea. Pigs became anorectic at the onset of diarrhea and grew lethargic within 12 h. Two pigs euthanized at 24 h PI were still strong, but two pigs were nearly moribund. Pigs weakened rapidly after this time. During the second 24 h period after inoculation, 13 of the 15 remaining pigs succumbed to the infection. Ten of these pigs were euthanized because they were moribund. Three pigs died between observation times. The last two pigs were very weak when euthanized at 72 hours PI.

All 15 pigs inoculated with both rotavirus and enterovirus developed watery diarrhea 18 to 24 h (mean 22.8 h) PI. Three pigs euthanized at 24 h PI were strong and alert. The three pigs euthanized during the second 24 h period after inoculation were weak and depressed. No pigs became moribund during this period, and only two pigs died. One of these died acutely of volvulus of the small intestine. From 49 to 72 h, five pigs were euthanized, but only two of these pigs were moribund. An additional moribund pig was euthanized at 88 h. The last pig was weak, but mobile at 96 h PI.

NECROPSY

The small intestines of all control pigs were thick-walled and contracted. Contents were watery, clear, and bright yellow with flecks of slimy yellow curd. Chyle was visible in lymphatics coursing over the intestinal serosa and through the mesentery. This feature was limited to the cranial one-third to two-thirds of the small intestine, and the villi and intestinal wall in these areas were pale. Mesenteric lymph nodes draining these portions of intestine were glistening white. Colon contents were firm to semisolid. Intestinal tracts of all enterovirus-inoculated pigs were similar to those of control pigs.

The small intestines of 15 of 19 pigs inoculated with rotavirus were thin-walled, dilated, and without tone. The small intestines of four pigs were slightly thicker-walled and more contracted, but were thinner-walled and more flaccid than the intestines of control pigs. The intestines in 12 pigs contained pale grey to yellow watery fluid with white flecks of milk curd, and in four pigs, a pale yellow homogenous thick liquid. In three pigs, intestines were congested and contents were watery and blood-tinged. There were membranous fragments of fibrinonecrotic debris adhered to the mucosa in two of these intestines. No chyle was visible in lymphatics or mesenteric lymph nodes. Gross lesions were not observed in the colons, but contents were fluid. There was impaction of the apex of the spiral colon in three pigs.

The small intestines of three of 15 pigs given rotavirus and enterovirus were thin-walled and flaccid. Intestines in five pigs were slightly thicker-walled and more contracted. In two pigs, intestines appeared similar in wall thickness and tone to those of control pigs. In five pigs, there was obvious variation in the condition of different segments of the small intestine. Intestinal contents in 12 pigs were watery, and in three pigs, the contents were of a thick liquid consistency. There was no hemorrhage or necrosis in any pigs. No chyle was visible in mesenteric lymphatics. No lesions were observed in spiral colons.

Gross lesions were not observed in organs other than the intestinal tract in any pigs.

HISTOPATHOLOGY

Microscopic lesions were not observed in stomach, colon, mesenteric lymph node, spleen, kidney, or brain from any pigs. There was mild focal interstitial pneumonia in the lungs of seven enterovirus-inoculated pigs.

Fig. 2. Relative lengths (4+ to 0) of villi at six levels of small intestine in pigs inoculated with rotavirus or enterovirus. Levels at which villous lengths were evaluated in each pig were (from left to right) duodenum, upper jejunum, middle jejunum, lower jejunum, upper ileum, and lower ileum. Number beneath each set of bars indicates time postinoculation at which pig was euthanized.

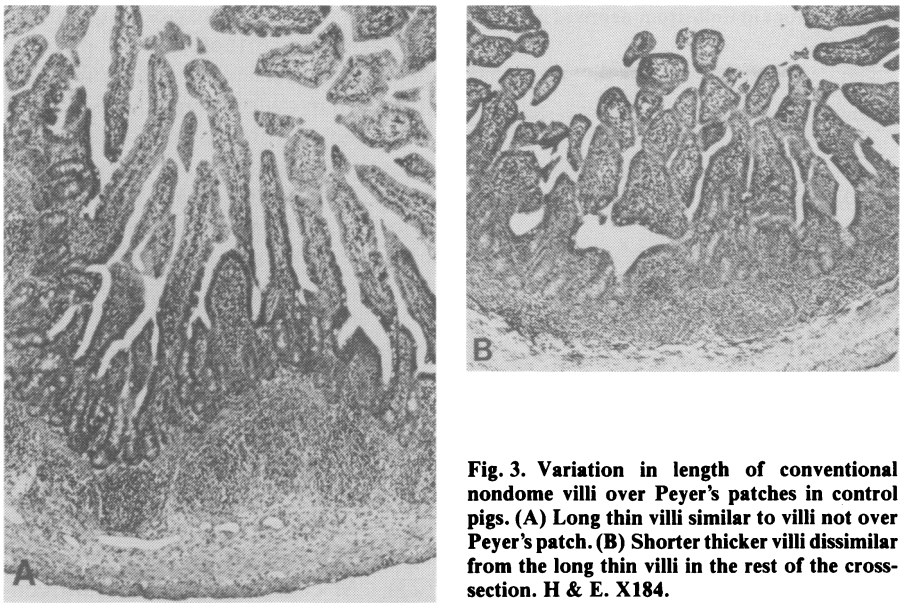

Fig. 3. Variation in length of conventional nondome villi over Peyer's patches in control pigs. (A) Long thin villi similar to villi not over Peyer's patch. (B) Shorter thicker villi dissimilar from the long thin villi in the rest of the cross-section. H & E. X184.

In intestines of control pigs, the longest villi and shallowest crypts were in the jejunum (Fig. 2). The shortest villi were in the ileum, but these villi were still very long and thin. Conventional or nondome villi over Peyer's patches were as long as or only slightly shorter than similar villi not over Peyer's patches (Fig. 3A). Dome villi, those villi containing a core of lymphoid cells that extended from the underlying submucosal lymphoid patches, were one-fourth to one-third the length of adjacent conventional villi. The lamina propria of villi in all segments consisted of very few cells in pigs up to 72 h old. There were increased numbers of reticular and endothelial cells and infiltrating lymphocytes in pigs older than three days. Conventional villi were slightly shorter over the Peyer's patch than in the rest of the section in one 120 h old pig (Fig. 3B). Dome villi in this area were not significantly affected.

The small intestines of pigs inoculated with enterovirus resembled those of control pigs (Fig. 2). Lesions were not observed. Conventional villi over Peyer's patches were slightly shorter in four pigs 96 to 120 h old. There were minimal changes in dome villi in these areas.

Villi in the small intestines of all 19 pigs inoculated with rotavirus were stunted. The severity and extent of villous destruction varied between pigs (Figs. 1 and 2), but all three isolates appeared equally virulent. Slight to moderate villous stunting affecting both jejunum and ileum was present in pigs euthanized at 24 h PI. Bulging and sloughing of villous epithelial cells were prominent. Neutrophils were clustered in the lamina propria at villous tips subjacent to disrupted epithelium. The most severe lesions were in five pigs that died or were euthanized 30-48 h PI. There was marked stunting throughout the jejunum and ileum in these pigs. Necrosis and sloughing of exposed lamina propria resulted in complete loss of villous structure in many segments. A thick layer of fibrinonecrotic debris was adhered to the exposed necrotic mucosa in two of these pigs. Severe lesions were localized to portions of small intestine in five other pigs that also became moribund or were euthanized 39 to 48 h PI. Villous stunting was more moderate in the two pigs that survived to 72 h. Stunting was distinctly more severe over the Peyer's patch than in the rest of the cross-section in some segments (Fig. 4). Both conventional and dome villi were affected.

Intestinal villi were stunted in all pigs inoculated with both rotavirus and enterovirus, but damage was not as extensive as in rotavirus-inoculated pigs (Fig. 2). There was complete destruction of villi throughout most of the small intestine in only one pig. In eight of the dual-infected pigs, severe stunting was restricted to portions of the jejunum with the ileum only moderately affected. In two pigs, villi were of moderate length throughout the small intestine. In one pig, villi were moderately stunted except for one portion of ileum, and in one pig, there were severely stunted villi in ileum and lower jejunum. Crypt hyperplasia and villous fusion were more prominent in dual-infected pigs than in pigs inoculated only with rotavirus. There was more severe stunting of both conventional and dome villi over Peyer's patches in five pigs 72 to 96 h old.

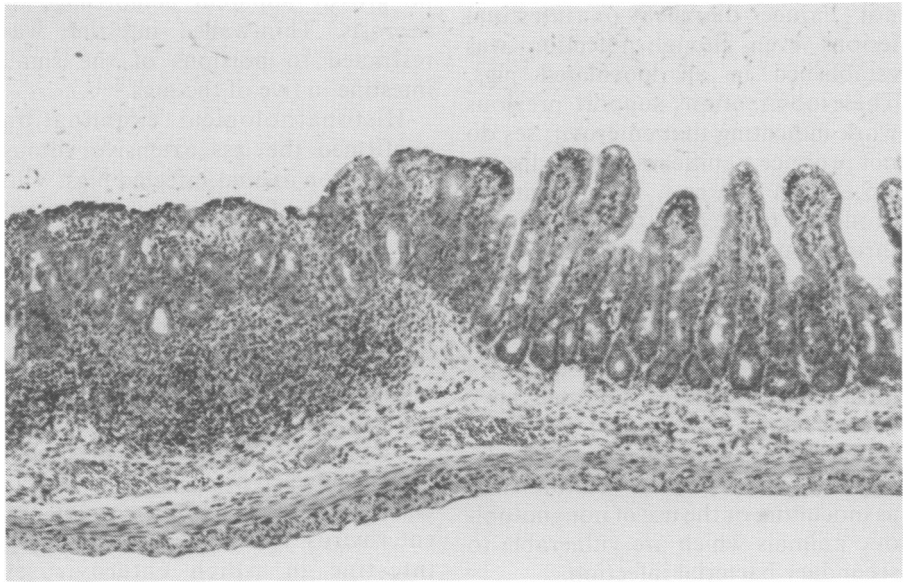

Fig. 4. More severe stunting of villi over the Peyer's patch in a rotavirus-inoculated pig. H & E. X184.

TABLE I. Mean Villous Length Scores for Each Level of Intestine in Colostrum-deprived Neonatal Pigs Inoculated Orally with Rotaviruses and Enteroviruses

Inoculum	Duodenum	Upper Jejunum	Middle Jejunum	Lower Jejunum	Upper Ileum	Lower Ileum
Control	3.3	3.9	4.0	3.8	3.3	3.0
Enterovirus	3.3	3.7	3.8	3.5	3.2	3.0
Rotavirus	2.9	1.4	1.5	1.3	1.5	1.4
Enterovirus and rotavirus	3.0	1.9	1.5	1.4	1.8	2.2[a]

[a]Mean villous length score for the lower ileum was significantly higher in rotavirus- and enterovirus-inoculated pigs than in rotavirus-inoculated pigs ($P < 0.01$)

Mean villous length scores for all pigs are summarized in Table I. Villous lengths in enterovirus-inoculated pigs were similar to those in control pigs. There was a marked reduction in villous length throughout the jejunum and ileum of pigs inoculated with rotavirus or rotavirus and enterovirus. Villous length scores were reduced similarly throughout the jejunum and ileum in rotavirus-inoculated pigs. However, in rotavirus- and enterovirus-inoculated pigs, villous length scores were higher in the upper jejunum and in the ileum than in the middle and lower jejunum. The difference in the mean villous length in the lower ileum between rotavirus-inoculated pigs and rotavirus- and enterovirus-inoculated pigs was statistically significant ($P < 0.01$).

DISCUSSION

The enteroviruses in this study did not induce diarrhea or intestinal lesions even though infection was established in all inoculated pigs. These observations support previous work indicating that enteroviruses do not produce significant enteric disease (15,16). In contrast, the rotaviruses produced marked destruction of villi throughout the small intestine. This resulted in rapid dehydration and death of almost all of the pigs given rotavirus only. Clinical disease was more severe than that observed in most previous studies. This may have been a reflection of the young age at which the pigs were inoculated, the use of pancreatin-treated cell culture virus as inoculum, or the use of nongnotobiotic animals which are vulnerable to secondary bacterial infection.

The length of incubation period before the onset of diarrhea in pigs inoculated with both rotavirus and enterovirus was similar to that in pigs inoculated only with rotavirus, but dual-infected pigs were affected less severely. By 48 h PI, 12 of 19 pigs inoculated only with rotavirus were moribund and three had died, while none of the dual-infected pigs had become moribund or died by this time. Only two rotavirus-inoculated pigs survived longer than 48 h, but nine of 15 rotavirus- and enterovirus-inoculated pigs survived beyond this time.

The moderation in clinical signs in dual-infected pigs appeared to be the result of less extensive intestinal damage than that observed in pigs infected only with rotavirus. The entire small intestines of 15 of 19 rotavirus-inoculated pigs were thin-walled and atonic, and hemorrhage or necrosis were noted in three pigs. Dramatic thinning of most of the small intestine was observed in only three dual-infected pigs, and there was no grossly apparent hemorrhage or necrosis. Thin-walled intestine was restricted to portions of the small intestine in five of the pigs.

Histopathological examination confirmed the less extensive villous destruction in dual-infected pigs. Villi in the ileum frequently were stunted less severely. The prominent crypt hyperplasia and villous fusion in dual-infected pigs was the result not only of injury that was less severe, but also of prolonged survival times which allowed regenerative changes in the intestines of these pigs.

The reduction in severity of disease in dual-infected pigs suggests interference with rotavirus infection by the enterovirus. Although the cells in pig intestine in which enteroviruses replicate have not been identified, villous epithelial cells do not appear to become infected. Previous studies on enterovirus infection in porcine nervous tissue (18) and poliovirus infection in cynomolgus monkeys (19) have shown that enteroviruses infect endothelial cells in brain and intestine. Once enterovirus has crossed the intestinal epithelium in the pig, it very likely replicates in endothelial cells of the lamina propria. After oral inoculation, enteroviruses are found initially in tonsil and in all levels of the intestinal tract. However, higher titers of virus are found in the ileum and colon, and virus persists in these areas for several weeks (20-22). Seventy-five percent of the submucosal lymphoid tissue in the small intestine of the pig is in one continuous patch in the ileum (23). M-cells, specialized antigen-transport cells located in the epithelium covering dome villi over Peyer's patches (24), may be the means by which enteroviruses traverse the intestinal epithelial barrier, such as has been reported for reoviruses in mice (25). Active uptake of enterovirus by M-cells would account for the higher concentration of virus in the ileum. Interferon can be induced within hours of infection and by diffusion from infected cells has its greatest protective effect on adjacent cells (26). Interferon from enterovirus-infected lamina propria endothelial cells may have reduced the number of villous epithelial cells in the ileum that were infected by rotavirus. This would account for the relative sparing of villi in the ileum in dual-infected pigs.

The greater stunting of villi over Peyer's patches in some rotavirus-inoculated pigs also may have been due to additional uptake of rotavirus by M-cells with subsequent heavier infection in these very localized areas. This greater stunting over Peyer's patches may represent an amplification of a normal nonspecific response of villi in these areas to antigenic material in the intestinal lumen. Although conventional villi over Peyer's patches were as long as the corresponding villi not over Peyer's patches in newborn pigs, preferential stunting of these villi has occurred in control and enterovirus-inoculated pigs by 48 h of age. Shortening of these villi was not as severe as in rotavirus-inoculated pigs. Why non-

pathogenic organisms or noninfectious material in the intestine would cause such shortening has not been addressed.

In summary, rotaviruses alone produced severe diarrhea in young pigs through widespread villous destruction whereas enteroviruses did not induce enteric disease. Oral inoculation with both rotavirus and enterovirus resulted in some reduction of rotavirus-induced villous damage. There was a consequent moderation in clinical signs and survival was prolonged in dual-infected pigs. These results suggest an inhibition of the rotavirus infection by the enterovirus. The mechanism of interference remains to be studied.

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