

Age-Dependent Resistance to Transmissible Gastroenteritis of Swine

III. Effects of Epithelial Cell Kinetics on Coronavirus Production and on Atrophy of Intestinal Villi

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Abstract. Coronavirus titers in small intestine, degree of villous atrophy and apparent rates of regeneration of intestinal villi were compared in newborn, 3-week-old and adult pigs for 1 week after they were exposed to the transmissible gastroenteritis virus of swine. The response within the newborn group was homogeneous, resulting in high virus titers, maximal villous atrophy and comparatively slow regeneration. In general, virus titers were lower, villous atrophy was less severe and regeneration more rapid in both older groups than in the newborn pigs. However, the response varied greatly in the older groups. The 3-week-old group was divided into two populations. The major population had low virus titers and developed partial villous atrophy, whereas the minor population had marked villous atrophy and virus titers comparable to those of the newborn pigs. These observations support the hypothesis that the accelerated replacement of villous epithelium in the small intestine of pigs during the first 3 weeks contributes to the innate age-dependent resistance to transmissible gastroenteritis. The accelerated replacement of villous epithelial cells in older pigs contributes to resistance in two ways. The increased proliferative capacity of crypt epithelium results in a more rapid regeneration of atrophic villi; and the comparatively young villous absorptive cells resulting from accelerated replacement produce less virus per cell than the older ones of the newborn pig.

Transmissible gastroenteritis of swine is an acute diarrheal disease caused by destruction of absorptive epithelial cells of the intestinal villi by a specific coronavirus. The resultant lesion is called villous atrophy. Crypt epithelium is not affected directly by the virus, and intestinal villi rapidly regenerate in pigs that survive the acute diarrheal phase of the disease [16]. Acute viral diseases of the small intestine in several species follow a similar sequence of selective cell destruction, leading to variable degrees of villous atrophy and diarrhea, followed by rapid regeneration of villi and the return of normal function. Epizootic diarrhea of infant mice [1], lethal intestinal virus infec-

tion of mice [3] and diseases caused by a coronavirus [12] and a reovirus-like agent [11] in young calves follow this pattern as does the human disease caused by Norwalk agent [2].

Pigs of all ages are susceptible; however, the frequency, severity and duration of diarrhea in newborn pigs is greater than in 3-week-old pigs exposed to the same virus [16]. The fatality rate is high in newborn pigs (approximately 100%) and low (usually less than 5%) in those older than 3 weeks. Because the signs and lesions result from the loss of villous absorptive cells in the small intestine and because 3-week-old pigs normally replace these cells more rapidly than newborn pigs [13], we hypothesized that age-dependent resistance was caused by more rapid regeneration of villi in the older pigs. In a test of this hypothesis [16], comparison of age groups according to regeneration rate was frustrated by the heterogeneity among 3-week-old pigs. This age group seemed to show two populations, one with and the other without villous atrophy. Interpretation further was complicated because virus titers were higher in newborns than in 3-week-old pigs [17]. Therefore, our initial hypothesis was modified: accelerated replacement of villous epithelium occurring in the small intestine of pigs during the first 3 weeks results in decreased virus production in the small intestine plus a more rapid regeneration of villi, contributing to the innate age-dependent resistance to transmissible gastroenteritis that develops during this period.

The major objective of this study was to compare coronavirus titers in small intestine, degree of villous atrophy and apparent rates of regeneration in newborn, 3-week-old and adult pigs for 1 week after exposure to the transmissible gastroenteritis virus. Secondary objectives were to determine if, in contrast to newborn pigs, there are two populations of 3-week-old pigs, according to villous atrophy and virus titer after exposure, and if there are differences in virus titer between jejunum and ileum.

Materials and Methods

Figs. Sixty-nine hysterectomy-derived, colostrum-deprived newborn pigs were randomly assigned to two groups. One group was designated newborn (31 pigs) and the other designated 3-week-old (38 pigs). Ten hysterectomy-derived, colostrum-deprived pigs, at least 1 year old, were kept in isolation until used. Principals and controls were selected randomly from each age group. All pigs were raised in the laboratory in isolation to prevent inadvertent exposure to the virus. Principals were isolated from controls before exposure.

Exposure. The virus preparation was from the same stock previously used [16, 21]. Principals in the newborn group had a mean body weight of 1.16 kg at 3 days of age when

they each were given, intragastrically, 2.0 ml of a 10^{-4} dilution in phosphate-buffered saline. Principals in the 3-week-old group had a mean body weight of 2.77 kg at 21 days of age when they each were given 4.76 ml of a 10^{-4} dilution, intragastrically. Principals in the adult group had a mean weight of approximately 200 kg when they each were given 20 ml of a 10^{-2} dilution orally. Four pigs in each of the newborn and 3-week-old groups, and three pigs in the adult group were unexposed controls.

Necropsy. Randomly selected pigs were killed by parenteral overdose of either sodium pentobarbital or succinyl choline and necropsied at once. Three principals from the newborn group were killed at each 72-, 120-, or 168-hour interval after exposure. Three principals from the 3-week-old group were killed at each 12-hour interval from 24 to 120 h and at each 24-hour interval from 120 to 168 h. Principals from the adult group were killed at intervals from 24 to 168 h. Some of the pigs that died were examined in the same manner as those that were killed. However, data from those two groups were separated. Data from pigs that died were not used in the figures in this report.

Histologic Examination. Segments from four sites equidistant along the entire course of jejunum and ileum in each pig were removed at necropsy, fixed in formalin, embedded in paraffin, cut into sections 7 μ m thick, stained with hematoxylin and eosin and examined with a light microscope equipped with an ocular micrometer. Five well-oriented villi from each of the four sites in each pig were measured and the results combined into a single value for mean villous length for each pig. Age, time interval and exposure status of the pigs were not known by the examiner.

Virus Titration. The five separate parts of small intestine remaining after removal of segments for histologic examination were labeled from one to five. These parts were homogenized individually to make a 10% suspension in phosphate-buffered saline, and the virus titer of each was determined [17] by a plaque assay in tissue culture using an agar overlay system. Titer was expressed as the number of plaque-forming units per gram of homogenized intestine.

In addition, mesenteric lymph nodes from exposed pigs in the 3-week-old age group were examined for coronavirus. Part of mesenteric lymph node taken at necropsy from each pig was teased free of connective tissue, finely minced in phosphate-buffered saline, filtered through a Millipore pad (0.22 μ m) and inoculated into an established line of swine testis cells in Earle's liquid medium with Eagle's additives. After 1 week's incubation at 37°C under 5% CO₂, cultures with characteristic cytopathology were recorded as containing coronavirus.

Results

Controls in all three age groups remained clinically normal. Twenty-six of the 27 newborn principals had diarrhea by day 3 after inoculation, and 25% of the pigs in this group died by day 5. In comparison, only two of 21 3-week-old and one of five adult pigs had diarrhea by day 3, and none of the pigs in these two groups died.

Virus titer did not vary consistently from site to site in the intestine. Pigs with high titers tended to have high titers in all five parts of the intestine. For

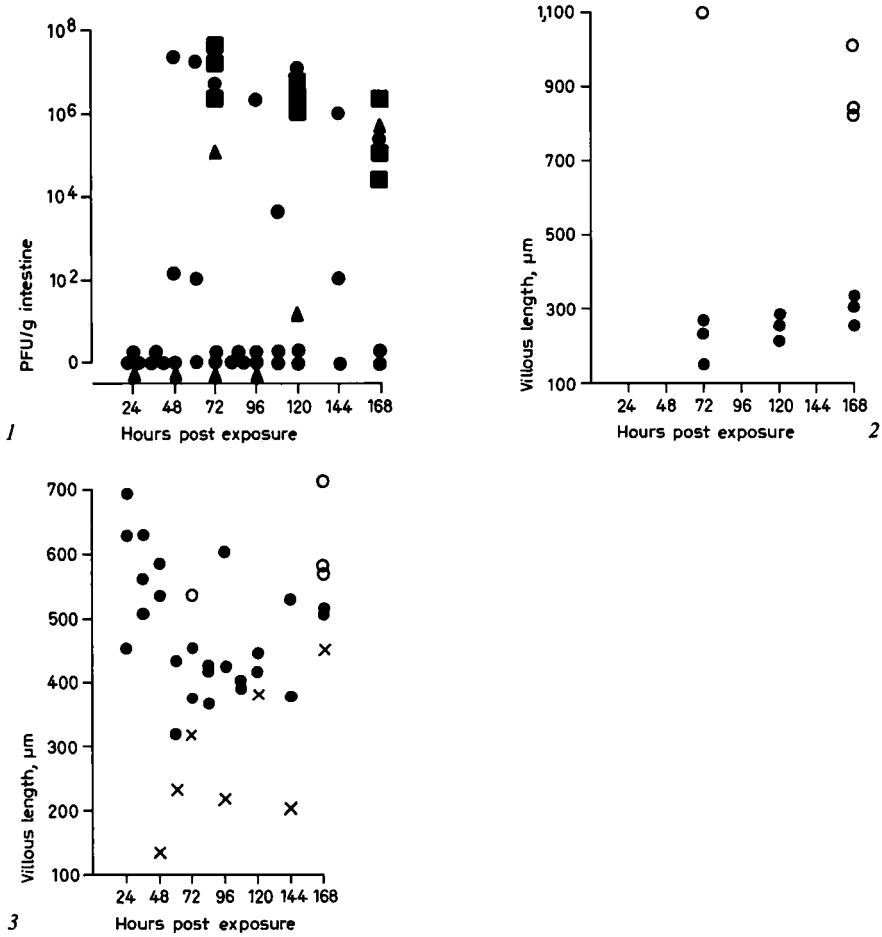


Fig. 1. Mean coronavirus titer of jejunum and ileum of pigs in three age groups exposed to coronavirus of transmissible gastroenteritis. Each mark represents results from one pig. Titer is the number of plaque-forming units per gram in a continuous line of swine testes cells in culture. ■ = 3-day-old group; ● = 3-week-old group; ▲ = adults.

Fig. 2. Mean length of villi in jejunum and ileum of pigs. Principals (●) were exposed to virus when 3 days old, controls (○) were the same age but not exposed. Each mark represents results from one pig.

Fig. 3. Mean length of villi in jejunum and ileum of pigs. Principals were exposed to virus when 3 weeks old, controls (○) were the same age but not exposed. Each mark represents results from one pig. Principals are divided into those more (×) and less (●) than 10⁵ plaque-forming units of virus per gram of small intestine at necropsy.

Table 1. Coronavirus titers of segments of small intestine from each of three pigs in three different age groups, killed 48–168 h after exposure to transmissible gastroenteritis virus

Intestinal segment ¹	48 h			72 h			120 h			168 h		
<i>3-day-old group</i>												
1	NE	NE	NE	5×10^6	5×10^7	6×10^6	4×10^6	1×10^6	1×10^6	3×10^3	1×10^4	2×10^4
2	NE	NE	NE	1×10^7	7×10^7	7×10^6	3×10^6	4×10^6	1×10^6	3×10^4	7×10^5	2×10^5
3	NE	NE	NE	2×10^7	6×10^7	5×10^6	4×10^6	2×10^6	2×10^6	8×10^3	7×10^5	3×10^4
4	NE	NE	NE	5×10^7	9×10^7	3×10^6	1×10^6	8×10^6	1×10^6	4×10^4	6×10^6	1×10^6
5	NE	NE	NE	6×10^7	4×10^7	3×10^6	6×10^6	4×10^6	7×10^5	1×10^5	4×10^6	8×10^5
<i>3-week-old group</i>												
1	0	4×10^2	9×10^7	0	0	1×10^6	0	0	3×10^5	0	0	0
2	0	2×10^2	2×10^7	0	0	1×10^7	0	0	2×10^7	0	0	4×10^4
3	0	3×10^2	6×10^6	0	0	1×10^7	0	0	9×10^6	0	0	6×10^5
4	0	5×10^1	2×10^7	0	0	5×10^6	0	0	7×10^6	0	0	6×10^5
5	0	0	3×10^6	0	0	8×10^6	0	0	2×10^6	0	0	8×10^5
<i>Adults</i>												
1	0	NE	NE	0	3×10^5	NE	0	NE	NE	1×10^1	NE	NE
2	0	NE	NE	0	3×10^4	NE	0	NE	NE	9×10^4	NE	NE
3	0	NE	NE	0	2×10^4	NE	0	NE	NE	2×10^5	NE	NE
4	0	NE	NE	0	7×10^2	NE	0	NE	NE	3×10^5	NE	NE
5	0	NE	NE	0	2×10^5	NE	6×10^1	NE	NE	3×10^5	NE	NE

NE = Not examined; 0 = coronavirus not detectable.

¹ Segment 1 is proximal jejunum, segment 5 is distal ileum, segments 2, 3 and 4 are between 1 and 5.

this reason, a single arithmetic mean virus titer of all parts was calculated and plotted for each pig (fig. 1); however, the titer of site 5 exceeded that of site 1 by more than 1 log in three of nine newborn pigs, six of 33 pigs 3 weeks old and two of eight adults. Conversely, the titer of site 1 exceeded that of site 5 by a log or more in three of 33 pigs 3 weeks old. Titers of individual sites for all pigs killed after 48, 72, 120 and 168 h are listed in table I. Some of the variations mentioned above are apparent by comparing 3-week-old pigs at 48 h after exposure to 3-day-old pigs at 168 h.

Titers in the newborn pigs were uniformly high and tended to decrease slightly from 72 to 168 h. Plaque-forming virus titers in the intestine of three newborn pigs that died after 72–96 h were comparable (10^6 – 10^7 /g) to those of pigs in this group that were killed during this interval. In contrast, titers of older pigs were not uniform. Virus was not detected in small intestine of

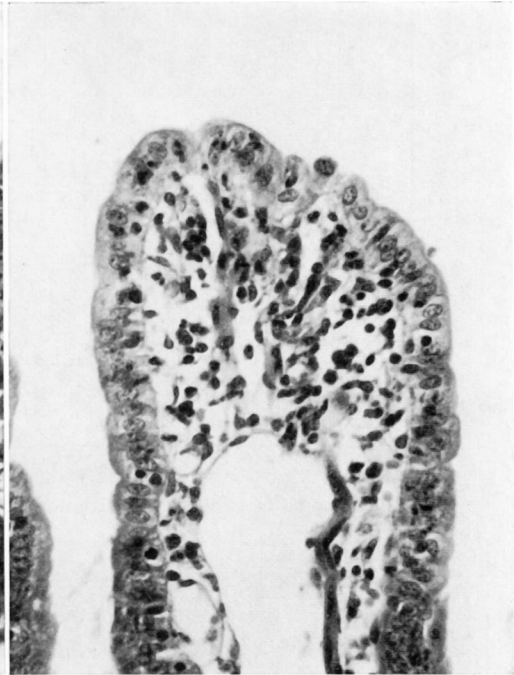
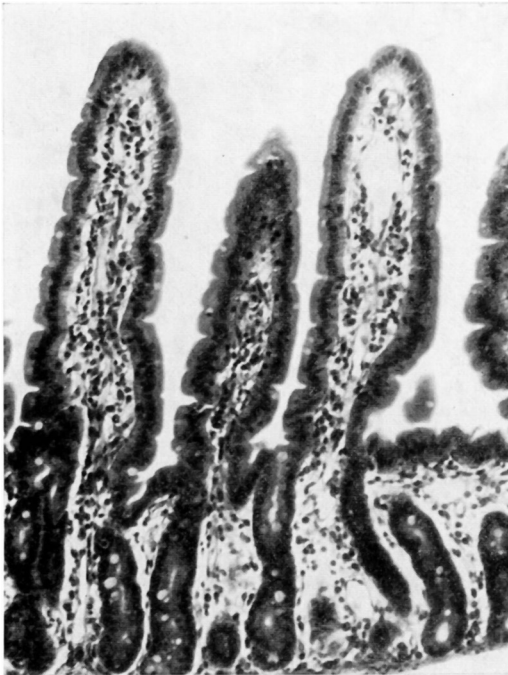


Fig. 4. Normal mucosa from the small intestine of a 3-week-old control pig.

Fig. 5. Epithelium of a partly atrophic villous in the small intestine of a 3-week-old pig with experimental infection. The small intestine of this pig contained insufficient virus to be detected by the tissue culture plaque assay. Epithelial degeneration, flattened, exaggerated sloughing and infiltration with inflammatory cells, along with decreased villous length are evidence of viral-induced mucosal damage.

most pigs from the 3-week-old and adult groups; a few had low titers and others had titers comparable to those of the newborn pigs (fig. 1). Virus was recovered from most of the 3-week-old pigs by inoculation of mesenteric lymph node into liquid cell culture. For example, all six lymph nodes from 3-week-old pigs taken 24–36 h yielded virus; however, none was detected by the intestinal titrations (done in a different system and at a different concentration) from these pigs (fig. 1).

The degree of villous atrophy among newborn principals was uniform at each interval, and villi regenerated slightly between 72 and 168 h (fig. 2). The lengths of the villi in five newborn principals that died from 72 to 96 h were comparable to those shown for the newborn principals that were killed. Three-week-old principals differed from the newborn in that villous atrophy

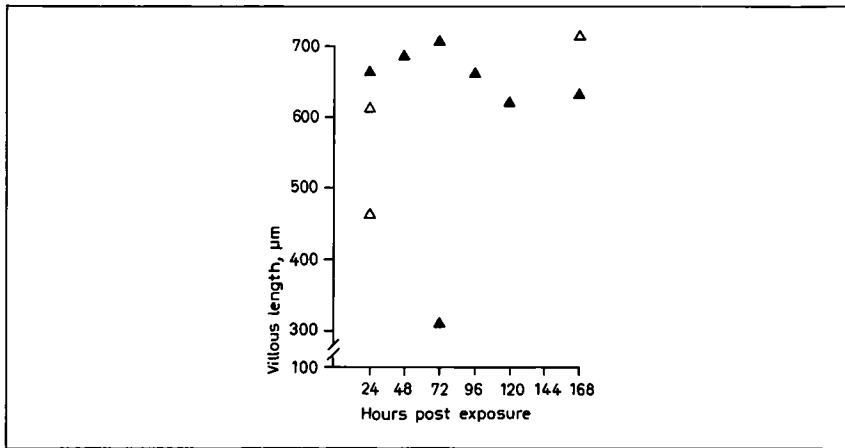


Fig. 6. Mean length of villi in jejunum and ileum of adult swine. Principals (▲) were exposed to virus, controls (△) were not. Each mark represents results from one pig.

tended to be less severe and to be markedly varied among pigs at 48–144 h (fig. 3). Furthermore, apparent regeneration of villi was more nearly complete by 168 h in the 3-week-old than in the newborn pigs.

The degree of villous atrophy in 3-week-old principals from which virus was not recovered, or was recovered in only comparatively low titer ($<10^5$), was consistently less than in pigs with high virus titers ($>10^5$) killed at the same intervals (fig. 3). An analysis of covariance for villous length was conducted on all 3-week-old principals; virus titer was used as a covariate. The analysis indicated that titer and villous length were significantly ($P < 0.01$) related; for example an increase in virus titer corresponds to a decrease in villous length. An analysis (t-test) of villous length in 3-week-old principals from which virus was not recovered also indicated that villous length in these pigs did decrease significantly ($P < 0.01$) from 24 to 60 h (fig. 1, 3–5).

Villous atrophy was detected in only one of the 10 adults (fig. 6, 7). This was the only adult that developed diarrhea and was the adult from which virus was recovered at 72 h (fig. 1).

Discussion

HOOPER and HAELTERMAN [8] found that pig duodenum and jejunum produced more transmissible gastroenteritis virus than did the ileum. We used a different experimental design, and virus titers did not vary consistently

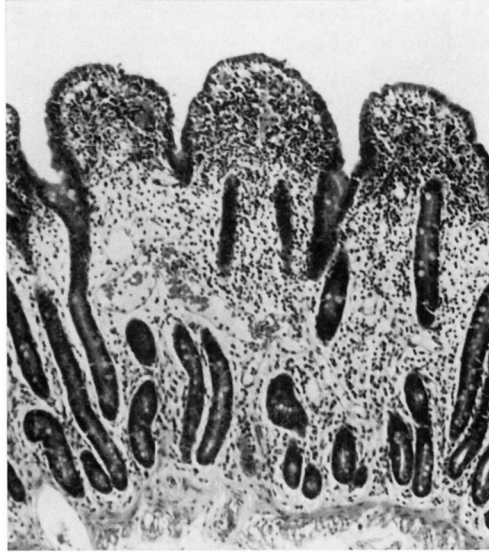


Fig. 7. Mucosa from the small intestine of an adult pig with experimental infection. Villi are atrophic and covered by a degenerate flattened epithelium. There is edema and increased inflammatory cell infiltration in the lamina propria. Crypt epithelium is not affected directly but undergoes compensatory hyperplasia.

with site in the intestine. In our study, some virus contained in the ileum could have been produced in the duodenum or jejunum. Virus production and villous atrophy of 3-week-old and adult pigs were less than in newborn pigs. These age differences probably contribute significantly to the innate, age-dependent resistance to transmissible gastroenteritis.

We did not detect virus by the plaque (titration) assay of intestine from most of the 3-week-old principals, but most did yield virus after inoculation of undiluted suspension of mesenteric lymph nodes into cell cultures in liquid media. Furthermore, pigs from which intestinal virus was not recovered developed partial villous atrophy after 48 h. The degree of villous atrophy in pigs from which virus was recovered was directly and significantly related to virus titer. Presumably, virus was present in low titer in the intestine of some pigs, but not detected by plaque assay. Three-week-old pigs consist of at least two populations. The major population produces less virus than newborn pigs, resulting in only partial villous atrophy and little or no diarrhea. On the other hand, the minor population produces virus in amounts comparable to the newborn, resulting in marked villous atrophy expressed clinically as severe diarrhea.

The apparent rate of regeneration of villi in the minor population of 3-week-old pigs was more rapid than in the newborn pigs (fig. 2, 3). This is consistent with that part of the hypothesis predicting more rapid regeneration in older pigs, based on the observation that normal newborns replace villous epithelium in 7–10 days; whereas 3-week-old pigs do so in 2–4 days [13]. Presumably, regeneration was continuous and concomitant with destruction in both groups. Thus, comparatively rapid regeneration in 3-week-old pigs may partially explain why maximal villous atrophy was less in this group than in newborn pigs.

The intestine of newborn pigs contained 10^2 – 10^5 times more virus per gram than that of most 3-week-old pigs (fig. 1). The entire small intestine of a 3-week-old pig 1–7 days after inoculation weighs only about five times more than that of a newborn pig [17]. Thus, there was considerably less virus in the intestine of most 3-week-old principals than in newborn principals. Virus is produced in villous epithelial cells [18, 22]. Villi of 3-week-old pigs are 30–50% shorter than those in newborn pigs and thus contain fewer epithelial cells [13, 16, 24]. Assuming the total number of intestinal villi in pigs is constant from birth to adult as in some species [4, 5], reduced villous length would result in fewer total productive cells and some reduction in titer. However, there is some evidence that the number of intestinal villi does increase during the neonatal period in pigs [24]. The modest reduction in titer expected from the reduced length of villi (even if villi are constant in number) would be inadequate to explain the differences in virus titer between newborn pigs and the major population of 3-week-old pigs.

We speculate that the major reason for the differences in virus titer between age groups is related to differences in epithelial cell kinetics. The normal life span of villous absorptive cells in 3-week-old pigs (2–4 days) is less than in newborn pigs (7–10 days) [13]. Virus production in the comparatively old cells of newborn pigs was greater than in the comparatively young cells of 3-week-old pigs. Other lines of evidence indicate a direct relationship between virus production and cell age. *In vivo*, this virus replicates in the mature, differentiated, nonproliferating villous epithelial cells and spares the juvenile, proliferating epithelial cells of the crypts [18, 22]. Furthermore, absorptive cells destroyed by the virus are rapidly replaced by juvenile epithelial cells from the crypts [22, 23], and these juvenile cells are comparatively resistant to virus production after they have migrated onto the villi [18]. *In vitro*, 5-day-old monolayers of swine testis cells produce more than 10-fold as much virus as do 2-day-old monolayers of the same cell line [20]. This relationship between cell age and production of the coronavirus of

transmissible gastroenteritis of swine appears to be in contrast to the parvovirus of feline panleukopenia (infectious feline enteritis). Panleukopenia virus is produced at high titer in young, rapidly proliferating tissue culture cells, but not by aged cells in culture [9] and directly affects crypt, but not villous, epithelium in the small intestine [6, 10].

The virus enters and accumulates in the apical tubular and vacuolar system of villous absorptive cells of newborn pigs [25], from which it replicates by internal budding. This tubular-vacuolar system is extensive in newborn pigs but lacking or less well-developed in pigs more than 3 weeks old [14, 19]. It has been suggested [25] that virus production by absorptive cells of older pigs is limited by the inadequate tubular-vacuolar system, thus limiting cell damage and contributing to age resistance. The differences in virus titer and villous atrophy with age reported here are consistent with this. Our hypothesis relating cell age to virus production is also consistent with their suggestion because the tubular-vacuolar system is not developed until more than 4 days after synthesis of DNA [15].

The hypothesis relating cell age to virus production may also explain why there were two populations of 3-week-old pigs. At this age epithelial replacement times vary from 2 to 4 days. Pigs with replacement times of about 2 days would be expected to have lower virus titers than those with replacement times of about 4 days.

Adult response was comparable to that of 3-week-old pigs with marked heterogeneity, generally lower virus titers than newborn pigs and little or no villous atrophy. Epithelial replacement rates for the intestine of adult pigs are unknown. Presumably they are comparable to those for 3-week-old pigs, and the relationships (among the kinetics of epithelial replacement, virus production and regeneration of villi) discussed above also contribute to the resistance of this group. These relationships explain why diarrhea is more frequent, severe and prolonged in newborn than in older pigs with transmissible gastroenteritis. This more frequent, severe and prolonged diarrhea, along with the higher percentages of body and extracellular water and the incompletely developed renal fluid and electrolyte regulatory mechanisms characteristic of the mammalian neonatal period probably account for the high fatality rate in newborn pigs.

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