SMALL INTESTINAL LESIONS OF TRANSMISSIBLE GASTROENTERITIS IN GNOTOBIOTIC PIGS: A SCANNING ELECTRON MICROSCOPIC STUDY

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ABSTRACT

SMALL INTESTINAL LESIONS OF TRANSMISSIBLE GASTROENTERITIS IN GNOTOBIOTIC PIGS: A SCANNING ELECTRON MICROSCOPIC STUDY

By

David P. Olson

Twenty-nine gnotobiotic pigs from 3 crossbred gilts were utilized to study the progressive morphologic changes in the small intestine following oral exposure to transmissible gastroenteritis (TGE) virus at 9 days of age. Pigs were euthanatized at 6, 12, 15, 18, 24, 36 or 48 hours after exposure. Comparisons were made between histologic and scanning electron microscopic lesions and clinical signs.

Clinical signs and gross lesions typical of TGE were observed in the exposed pigs from 2 of the 3 litters. Gross lesions were observed in the digestive tracts of all pigs euthanatized 12 hours or more after exposure. Diarrhea was observed in pigs as early as 15 hours after exposure.

The normal intestinal structures and lesions observed by light microscopy correlated well with the structures and lesions observed by scanning electron microscopy. Early subgross and microscopic changes in the small intestinal villi and related structures were observed beginning at 15 hours after exposure. They included shortening of villi, deepening of the transverse furrows and partial exposure of the circumvillar basins and crypts of Lieberkühn. There was uniform and consistent atrophy of all the villi in the jejunum and ileum by 18 hours.

Metaplasia of the epithelium covering the villi was obvious by light microscopy. The epithelium became vacuolate and sloughed from the surface of many villi so that almost total denudation had occurred by 36 hours. Complete atrophy of many villi and total exposure of the circumvillar basins were observed by 48 hours. Villous atrophy and damage to the surface epithelium appeared to be more extensive by scanning electron microscopy. There was some variation in the extent of the subgross and microscopic lesions in exposed pigs euthanatized at the same time after exposure.

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David P. Olson

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INTRODUCTION

Enteric diseases of domestic animals result in major economic losses to the animal industry. Transmissible gastroenteritis (TGE) in swine is a viral disease which is particularly devastating in terms of high mortality in young pigs less than 2 weeks of age. Continued research on TGE is necessary to determine the pathogenesis and to devise methods for the prevention and control of the disease.

Experience in clinical practice has helped the author generate and maintain a special interest in further study and research on TGE. The scanning electron microscope (SEM) has recently evolved as a useful means of studying normal and abnormal biological tissues. As a result, the SEM was used in the present study to characterize and describe the lesions which occur in the small intestine of gnotobiotic pigs experimentally exposed to TGE virus.

OBJECTIVES

The objectives of this research were:

1. To experimentally reproduce TGE using 9-day-old gnotobiotic pigs in a controlled study.

2. To prepare sections of small intestine from exposed and unexposed pigs at specific sites and at selected time intervals within 48 hours following exposure.

3. To describe and compare the lesions involving the small intestinal villi and related structures using the light microscope and the SEM.

REVIEW OF LITERATURE

Normal Anatomy of the Small Intestinal Mucosa

Villi. The absorptive surface area of the small intestine is markedly increased by the formation of circular folds (valves of Kerkring) and numerous conical outgrowths of the mucous membrane known as villi (Maximow and Bloom, 1952). Villi measure approximately 0.5 to 1.0 mm. in length and 0.2 mm. in width (Trautman and Febiger, 1952). Pfeiffer (1958) reported a wide variation of normal villous architecture among various animal species, among different age groups and within the same and different regions of the intestinal tract.

> "Also, there are 4 general forms of villi: (1) finger shaped, (2) threadlike, (3) leaf shaped, and (4) low wart-like. These forms represent the range of structures found in vertebrates."
> (Pfeiffer, 1968)

The number of villi in pigs is believed to increase during embryonal life and also from birth to adult age (Pfeiffer, 1968). Normal contraction of smooth muscle bundles causes shortening of villi and formation of circular folds of epithelium (Trautman and Febiger, 1952). The innumerable openings of the tubular glands of Lieberkühn are located between the bases of the villi and penetrate the mucous membrane (Maximow and Bloom, 1952).

Epithelium. The mucous membrane of the small intestine is composed of 3 cell types: (1) columnar cells with a striated border, (2) goblet cells, and (3) argentaffin cells. The columnar cells are prismatic in

form and change shape with normal movement of the villi (Maximow and Bloom, 1952). Sibalin and Bjorkman (1966) described the gross and ultrastructural features of jejunal epithelium from normal pigs.

Autoradiographic techniques have proven the dynamic histologic concept that absorptive epithelial cells are continually being produced and replaced in the intestine (Padykula *et al.*, 1962). These cells originate in the crypts of Lieberkühn and migrate steadily upward along the sides of the villi to the apex. There they reach a distinct cleft on the surface called the extrusion zone. The cells are squeezed out of position at this zone and extruded into the intestinal lumen. The movement along the villi has been measured radiographically by incorporating tritiated thymidine into the migrating cells. The cytoplasm of undifferentiated crypt cells is high in ribonucleoprotein which is basophilic. The cell cytoplasm becomes more acidophilic as migration continues (Padykula *et al.*, 1962).

Moon (1971) used autoradiographic technique and reported that small intestinal epithelium in normal day-old pigs is replaced in 7 to 10 days, whereas the replacement time in 3-week-old pigs is 2 to 4 days.

Goblet cells originate from the crypts and are interspersed between absorptive epithelial cells. They empty their mucinous contents into the intestinal lumen (Trautman and Febiger, 1952). They may be more abundant in the crypt area (Trautman and Febiger, 1952) and relatively absent towards the tips of villi (Padykula *et al.*, 1962).

Microvilli. The entire luminal surface of the intestinal epithelium is covered by finger-like projections known as microvilli. Microvilli are short and irregular in the crypt cells (Thake, 1968) and become longer, narrower, and more numerous as the cells mature. The average

height is 1.36 μ and the average diameter is 0.08 μ. As the absorptive cells differentiate, there is an increase in the number and size of microvilli and a subsequent increase in surface area (Padykula *et al.*, 1962). An extraneous coat (Sibalin and Bjorkman, 1966) or glycocalyx (Thake, 1968) has been described covering the surface of the microvilli.

Transmissible Gastroenteritis in Pigs

History. The first report of an enteric disease in baby pigs characterized by vomiting, diarrhea, and high mortality was recorded in Indiana by Doyle and Hutchings (1946). What they observed and documented was the first accurate description of TGE. Whitehair *et al.* (1948) similarly reported on a gastroenteric disease resulting in almost 100% morbidity and 70 to 90% mortality in young suckling pigs. They were unable to isolate and demonstrate any pathogenic organisms, and the lesions noted were found primarily in the small intestine. Another early report (Young and Underdahl, 1947) involved an epidemiological study of a disease affecting newborn and older pigs. The clinical signs and mortality observed were strongly suggestive of TGE. The review by Smith (1956) reported other early investigations of the disease. Feenstra *et al.* (1948) studied TGE in baby pigs and compared it with a gastrointestinal disease, believed to be of viral origin, in human infants.

Transmissible gastroenteritis has been described in other countries according to Woode (1969).

Etiology. Doyle and Hutchings (1946) and Feenstra *et al.* (1948) reproduced TGE by inoculating ground intestinal contents, or bacteria-free filtrates, from infected into susceptible pigs. Bay *et al.* (1949)

reproduced the disease with dilutions of gastrointestinal filtrates as high as 1:1,000,000. The early work of Young and Underdahl (1947) supported the opinion that TGE is related to an infection. They noted signs of disease within 3 days, suggesting infection *in utero* or at birth. This study also indicated that nutrition, heredity, and environment play a secondary role in pathogenesis. The transmission studies by Whitehair *et al.* (1948) and Bay *et al.* (1949) suggested an etiologic agent other than a bacterial microorganism. Lee *et al.* (1954) made observations and studies with a viral agent isolated from 2 field outbreaks of TGE in New York State. Lee (1956) also initiated studies to propagate the TGE virus on pig kidney tissue culture and develop a suitable antigen for serologic testing. Young *et al.* (1955) reported the use of disease-free pigs in the study of TGE and determined virus particle size, age susceptibility, and susceptibility to infection by various routes of administration. These and other early studies finally confirmed the viral cause of the disease.

Bay *et al.* (1951) reported that the virus seems to have an affinity for the epithelium of the kidneys and the gastrointestinal tract. This was further supported by Hooper and Haelterman (1966a), who reported the primary site for virus replication was the epithelial cells of the duodenum and jejunum and, to a lesser degree, of the ileum.

Clinical Signs. The early reports of Doyle and Hutchings (1946) and Feenstra *et al.* (1948) described the clinical signs of TGE. The disease in newborn pigs is characterized by an incubation period ranging from 1 to 7 days with death occurring within 2 to 7 days following exposure. Principal signs include vomiting, dehydration, rapid weight loss, watery green to yellow and odorous feces, inappetence, slight early

temperature rise, extreme weakness, coma, and death. Mortality is usually 80 to 100% in pigs less than 2 weeks. Clinical signs in older pigs are usually transient and include diarrhea, vomiting, inappetence, agalactia in sows, low mortality, and recovery.

Gross Lesions. The gross lesions were described by early workers and later by Hooper and Haelterman (1966a, 1969) and Bohl (1970). They include evidence of dehydration and loss of body weight, distention of the stomach with milk curd, engorgement of the gastric and mesenteric blood vessels, and absence of chyle in the subserosal and mesenteric lymph vessels and nodes. Distention of the intestine with white to yellow-green fluid, urate deposits in the renal pelvis, and other degenerative changes in the kidney have also been described.

Atrophy of small intestinal villi was first described by Hooper and Haelterman in 1964 as an important lesion of TGE (Bohl, 1970). Neither malnutrition nor failure of mitotic activity appears to be the basis for the atrophy, however (Hooper and Haelterman, 1966b). The functional significance of villous atrophy was interpreted as a response to the rapid loss of mucosal epithelium (Hooper and Haelterman, 1966a; Thake, 1968) and may represent an attempt to maintain an intact epithelial covering (Hooper and Haelterman, 1966b; Trapp *et al.*, 1966; Bohl, 1970). Villous atrophy is also associated with a failure of cells, migrating upward from the crypts of Lieberkühn, to undergo normal differentiation and maturation into columnar epithelium (Hooper and Haelterman, 1969; Thake, 1968). According to Moon *et al.* (1970), villous atrophy with TGE is a primary factor in the pathogenesis of diarrhea. Trapp *et al.* (1966) found the most striking differences in atrophy between exposed and unexposed gnotobiotes to occur in the

jejunum and ileum, and not in the duodenum. The villi in the first few centimeters of the duodenum are either spared or show less severe changes (Haelterman and Hooper, 1967; Hooper and Haelterman, 1966b, 1969; Cross and Bohl, 1969; Bohl, 1970). These changes are also less severe in the ileum and in the villi located over Peyer's patches according to Hooper and Haelterman (1969). Hooper and Haelterman (1969) indicated that, within 1 day after exposure, most of the villi are atrophied to a uniform and maximum degree. Trapp *et al.* (1966) reported the absence of villi from some areas of the small intestine 72 hours after exposure.

Microscopic Lesions. Cellular degeneration in the small intestine with TGE has been described (Bay *et al.*, 1951; Trapp *et al.*, 1966; Hooper and Haelterman, 1969; Bohl, 1970). There are conflicting reports regarding the histopathologic changes which occur in the mucosal epithelium (Bohl, 1970). Only those changes which seem pertinent to this study will be discussed.

There is general agreement that epithelial cells are lost rapidly and in large numbers in TGE. The cells covering the shortened villi are undifferentiated and appear as low cuboidal or squamous type cells (Hooper and Haelterman, 1966b, 1969; Bohl, 1970). The affected villi are seldom denuded of epithelium (Hooper and Haelterman, 1966b; Haelterman and Hooper, 1967; Bohl, 1970). Trapp *et al.* (1966), however, reported the absence of villous epithelium in some areas of the small intestine in gnotobiotic pigs euthanatized 72 hours after exposure. Bay *et al.* (1951) also found denudation of epithelium involving the upper 1/3 to 1/2 of affected villi. Thake (1968) noticed that the tips of some villi were partially denuded.

Vacuolation of epithelial cells of the jejunum and ileum has been described as a finding in normal gnotobiotic pigs (Alexander *et al.*, 1969). Vacuolation of epithelial cells has also been described in pigs with TGE by Hooper and Haelterman (1969).

> "Vacuolation was observed in the epithelial cells of normal pigs, but rarely. When it occurred it was mostly in the cells at the tip of the villus. In the jejunum, vacuolation was present in a large number of intestines 1 day after infection and was present in almost all jejunal sections from the second through the fifth day after infection. In pigs which were recovering from TGE, the vacuolated cells were found primarily in tissues where villous atrophy was still marked. Epithelial cells in the ileum appeared less degenerate than those in the jejunum with cells positioned on villi located over lymphoid tissue having the least degenerate appearance."

Thake (1968) found many fat globules in the cytoplasm of epithelial cells from intestine infected with TGE.

Moon (1970) reported vacuolation of small intestinal epithelial cells in conventional pigs experimentally infected with several enteropathogenic strains of *Escherichia coli*. Christie (1967) and Drees and Waxler (1970) also noted vacuole formation in gnotobiotic pigs experimentally infected with *Escherichia coli* O138:K81:NM.

The microvillous border of intestinal epithelial cells of pigs with TGE is often reduced in height, indistinct, or absent (Hooper and Haelterman, 1966b, 1969; Maronpot and Whitehair, 1966; Thake, 1968; Trapp *et al.*, 1966). Thake (1968) also noted irregular spacing and arrangement of microvilli.

Thake (1968) reported no dilatation or alteration of the cellular interdigitations and junctional complexes of jejunal epithelium of pigs with TGE.

According to Trapp *et al.* (1966) and Hooper and Haelterman (1969), there is a normal ratio of villous-height/crypt-depth (V/C) of 7:1 in the small intestine. In TGE, the V/C ratio is reduced to 1:1 (Trapp *et al.*, 1966; Hooper and Haelterman, 1969). Tabulated data indicated that the abnormal V/C ratio resulted from shortening of the villi and lengthening of the crypts. Moon (1971), however, reported a similar situation in the normal animal by stating that a decreased epithelial cell replacement time in the intestine with age is associated with an increase in crypt depth, decreased villous length, and a resultant 3-fold decrease in V/C ratio.

According to Moon (1970), TGE is not the only common enteric disease of pigs which causes villous atrophy. Conventional newborn pigs were experimentally infected with several enteropathogenic strains of *Escherichia coli*.

> "Four of 18 pigs with enteric colibacillosis had segmental areas of villous atrophy in the mucosa of the small intestine. This lesion was also seen in a pig with experimental colibacillosis in an earlier study. However, villous atrophy has not been mentioned in previous reports of experimentally produced or naturally occurring enteric colibacillosis....The pathogenesis of villous atrophy observed in the present study is unknown. Villous atrophy is a nonspecific response of the intestine that occurs in many species and has a wide variety of causes and may or may not be associated with clinical disease." (Moon, 1970)

Other Related Intestinal Diseases

Villous atrophy is not limited to common enteric diseases in pigs. Thake (1968) reported that villous atrophy occurs in lethal intestinal virus infection of mice, a disease syndrome which is similar in many respects to TGE in infant pigs. Villous atrophy can also be produced experimentally by the administration of neomycin, by irradiation, and

by substances which inhibit mitosis. Collins (1965) and Pfeiffer (1968) reviewed the variety of clinical and experimental conditions in man associated with damage to the small intestinal mucosa and villi. These changes noted were not specific for any of the underlying disorders listed. Sheehy *et al.* (1964) stated,

> "Unfortunately, little is known about the enteropathic effects of viruses, even though the small intestine may serve alternately or jointly as host, victim, and perhaps even as incubator for certain viruses."

He further stated that there are few known studies of histopathological lesions of the gastrointestinal tract of man during the acute phases of viral illness. Curran and Creamer (1963) studied biopsy specimens of jejunal mucosa from human patients suffering from various malabsorption diseases. Padykula *et al.* (1961) used similar techniques in studying intraluminal biopsy specimens from human patients affected with non-tropical sprue. Crane (1966) proposed that some of the malabsorption diseases in humans may be a manifestation of brush border disease.

Similarities between TGE in pigs and celiac disease in man have been noted (Maronpot and Whitehair, 1966, 1967; Haelterman and Hooper, 1967). Detailed studies by Reber and Whitehair (1955) and Thake (1968) have monitored important changes in intestinal enzymes, electrolytes, and blood constituents of pigs with TGE. Haelterman and Hooper (1967) proposed that TGE be used as a model for basic studies of enteric diseases. They also stated that there are more physiological similarities between pigs and man than between carnivores and man.

Scanning Electron Microscopy

Introduction. The theoretical principles and techniques of scanning electron microscopy have been known since the 1930's, but the first

commercial SEM was not available until 1965 (Kavin *et al.*, 1970). Scanning electron micrographs were published, however, as early as 1942 (Hayes and Pease, 1968). The SEM is an electron optical advance of the dissecting microscope (Demling *et al.*, 1969) and provides visualization of the ultramicroscopic surface structure of specimens (Kavin *et al.*, 1970). The advantages of scanning electron microscopy over light or dissecting microscopy are greater resolution, increased depth of focus, and higher magnification. This provides a three-dimensional appearance of specimens observed (Kavin *et al.*, 1970; Asquith *et al.*, 1970). Many investigators (Cocco *et al.*, 1966; Marsh and Swift, 1969; Toner and Carr, 1969; Demling *et al.*, 1969; Kavin *et al.*, 1970; Asquith *et al.*, 1970) have used the scanning electron microscope to study the normal and abnormal intestinal mucosa.

Mechanical Principle of Scanning Electron Microscopy. The mechanical principle of the SEM has been described (Marsh and Swift, 1969; Kavin *et al.*, 1970; Hayes and Pease, 1968) and involves the emission of a high-energy, small-diameter electron beam from a tungsten filament. The beam passes over the specimen surface and causes emission of secondary low-energy electrons. The secondary electrons are then accelerated into a cathode-ray tube. The brightness of the image depends upon the angle at which the primary beam strikes the specimen. Darker images result when the specimen is perpendicular or a great distance from the beam, whereas brighter images result from a tangential specimen-to-beam relationship (Demling *et al.*, 1969).

Specimen Preparation. Various procedures have been described for the preparation of tissues for scanning electron microscopy (Toner and Carr, 1969; Marsh and Swift, 1969; Asquith *et al.*, 1970). All procedures

include fixation, complete drying, and coating with a thin layer of vaporized metal (Kavin *et al.*, 1970).

Scanning Electron Microscopic Description of the Intestinal Mucosa. The descriptions of the ultrastructural surface of the small intestine by Cocco *et al.* (1966), Toner and Carr (1969), Marsh and Swift (1969), Demling *et al.* (1969), and Asquith *et al.* (1970) have helped to clarify current understanding of the histologic orientation of that portion of the digestive tract.

Villi. Demling *et al.* (1969) and Marsh and Swift (1969) described the villi as finger- or leaf-shaped projections that are circular in cross section. The surface of many villi is interrupted by irregular furrows or corrugations which are of varying depth (Toner and Carr, 1969; Marsh and Swift, 1969; Asquith *et al.*, 1970) and are presumably caused by normal contraction or by tissue fixation (Demling *et al.*, 1969). Cocco *et al.* (1966) studied the three-dimensional relationship between the crypts and villi by reconstructing parallel and perpendicular serial sections of jejunal mucosa. Each villus is surrounded by a circumvillar basin which is formed by a series of vestibules. The surface of each vestibule is interrupted by 4 or more crypt openings (Demling *et al.*, 1969; Asquith *et al.*, 1970) and intercrypt ridges (Cocco *et al.*, 1966; Asquith *et al.*, 1970). Some villi are seen projecting through a network of mucous strands and membranes (Demling *et al.*, 1969).

Epithelium. The pattern of the epithelial cell outline is regular and hexagonal and resembles a honeycomb (Demling *et al.*, 1969; Marsh and Swift, 1969). Individual cells project from the surface in a hemispherical pattern and increase the absorptive surface by an additional 25% (Demling

et al., 1969). Toner and Carr (1969) suggested that shrinkage artifact may account for some of the hemispherical and hexagonal cell outlines. Trier and Rubin (1965) reviewed the present concepts of the ultrastructure of intestinal epithelium as they relate to absorptive and secretory functions.

Numerous pits on the surface of villi correspond to the openings of individual goblet cells. Mucus can be seen bulging from those cells that are discharging their contents (Toner and Carr, 1969). Undischarged goblet cells are difficult to identify because of a microvillar covering and a luminal plasma membrane similar to adjacent absorptive cells (Marsh and Swift, 1969).

Microvilli. Asquith *et al.* (1970) stated that the surface granularity of the absorptive cells is due to microvilli and Marsh and Swift (1969) demonstrated filamentous and rod-shaped microvilli. It may be difficult to demonstrate individual microvilli because of mucus and the coating of vaporized metal on the surface according to Demling *et al.* (1969).

Summary

Transmissible gastroenteritis has become established during the past 26 years as an economically important enteric disease affecting swine of all ages. The primary target organ of the TGE virus is the small intestine. Previous gross and histopathologic studies of the lesions of TGE have contributed to the present understanding of the pathogenesis of the disease.

Researchers have established the usefulness of scanning electron microscopy in studying the morphology of the normal gastrointestinal tract and also the lesions in certain malabsorption syndromes in humans.

Scanning electron microscopy may present a useful means of studying the lesions of TGE.

MATERIALS AND METHODS

Animals

Twenty-nine gnotobiotic pigs were derived from 3 crossbred gilts in the 111th or 112th day of gestation. Delivery by hysterotomy was performed according to techniques previously described (Waxler *et al.*, 1966). Anesthesia was achieved by epidural injection of 25.0 ml. of 2.5% procaine hydrochloride[a] at the lumbosacral articulation (Getty, 1963) followed by the intramuscular injection of 5.0 ml. of tranquilizer.[b]

The surviving pigs were aseptically transferred into plastic rearing isolators where they were divided according to sex and maintained until 9 to 11 days of age. The pigs were also divided into exposed and unexposed groups (Table 1).

All exposed pigs were kept in isolators, separate from the unexposed pigs. Each isolator contained a maximum of 4 pigs in individual cages along with equipment for feeding, inoculation, and sample collection for microbiologic determinations. An adequate supply of a commercial sterilized liquid diet[c] was aseptically transferred into each isolator, and a measured quantity was placed in pans at each feeding. The pigs were taught to drink from pans by submerging their noses and mouths.

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[a]Epidural, Haver-Lockhart, Kansas City, Mo.

[b]Sparine, Wyeth Labs, Philadelphia, Pa.

[c]SPF-Lac, Borden Company, New York, N.Y.

Table 1. Distribution of pigs from 3 litters

Litter No.	Exposed		Unexposed	
	Male	Female	Male	Female
1	2	4	1	3
2	4	1	1	1
3	4	3	2	-

They were fed and observed 3 times a day. The volume of liquid diet fed was approximately 3.0 oz. the first feeding and 4.0 oz. thereafter. The temperature of the rearing units was maintained at 32 C. for 3 to 4 days, then gradually reduced to 29 C.

Preparation and Administration of Inoculum

The TGE virus (designated No. 4658)[d] was obtained frozen in a sealed glass ampule and contained approximately 1 x 10^6 pig infective doses (PID)/ml. The virus containing material was rapidly thawed in a 37 C. waterbath and diluted 1:10 with Hank's BSS medium (Kalter, 1963). The medium was supplemented with 0.5% lactalbumin hydrolysate,[e] 0.05% TC yeastolate,[e] 100 I.U. potassium penicillin-G,[f] 100 µg. streptomycin sulfate,[g] and 50 I.U. polymxyin B sulfate.[f] The mixture was filtered through a 200 nm membrane filter.[h] The filtrate was transferred into

[d]Supplied by Dr. E. O. Haelterman, Purdue University, Lafayette, Ind.

[e]Difco Lab, Detroit, Mich.

[f]Pfizer and Co., New York, N.Y.

[g]E. R. Squibb and Sons, Princeton, N.J.

[h]Nalge, Subron Corp., Rochester, N.Y.

duplicate 5.0 ml. sterile glass ampules and heat-sealed. The estimated loss of viral titer from filtration was 1.4 logarithms (Sheffy, 1965) and the resulting final concentration was approximately 1 x $10^{3.6}$ PID/ml. Duplicate ampules containing sterile diluent were also prepared and heat-sealed.

The ampules containing the virus were aseptically transferred into the isolators, and each exposed pig was given a 1.0 ml. oral inoculum at 9 days of age. The virus was aspirated into a 5.0 ml. disposable plastic syringe and squirted into the pig's mouth. Each unexposed pig was given a 1.0 ml. inoculum of sterile diluent in the same manner. Observations for signs of illness continued following exposure until the time of euthanasia. Special effort was made to examine the pigs and the pans containing waste material for evidence of vomition.

Bacteriologic Procedures

Swabs were taken from each isolator prior to exposure of the pigs. Specimens consisted of rectal swabs and waste material from the cages. Material was streaked on tryptose blood agar[e] plates and inoculated into thioglycollate broth.[e] The media were incubated aerobically and anaerobically at room temperature, 37 C., and 55 C. Material was also inoculated into PPLO broth[e] and incubated aerobically at 37 C. for 5 days. A blind passage was then made from the broth onto a PPLO plate and into another broth and incubated in the same manner for 5 days. The same blind passages and incubation were repeated 2 more times. All cultures were observed for 3 weeks.

Collection of Intestinal Specimens

Pigs were euthanatized at 6, 12, 15, 18, 24, 36, and 48 hours after exposure (Table 2). Euthanasia was accomplished by injection of 1.5 ml.

Table 2. Number of exposed and unexposed pigs and time interval between exposure and euthanasia

Litter No.	Group	Interval Between Exposure and Euthanasia (hours) 6	12	15	18	24	36	48
1	Exposed	1	1	-	2	2	-	-
	Unexposed	1	1	-	1	1	-	-
2	Exposed	-	1	-	2	2	-	-
	Unexposed	-	1	-	-	1	-	-
3	Exposed	-	-	2	-	1	2	2
	Unexposed	-	-	-	-	-	1	1

sodium pentobarbital[i] in the anterior vena cava followed by exsanguination. They were then placed in dorsal recumbency, and the sternum and ventral abdominal wall were incised and reflected caudally exposing the abdominal viscera. The small intestine was removed by severing it adjacent to the pylorus and to the ileocecal valve and incising along the mesenteric attachment. The intestine was placed on a table in a "Z" pattern so that the 3 sections were approximately of equal length. Care was taken not to stretch or crush the intestine. Paired, adjacent full-thickness sections, approximately 2.0 cm. long, were cut at each of 4 locations. The first were taken from the duodenum starting 5.0 cm. from the pylorus and progressing caudally, and the last were taken from the ileum starting 5.0 cm. anterior to the ileocecal valve and progressing anteriorly. The middle sections (upper and lower jejunum) were taken 1/3 and 2/3, respectively, of the distance from the pylorus to

[i] Haver-Lockhart Lab, Kansas City, Mo.

the ileocecal valve. One of the paired sections was prepared for scanning electron microscopy and the other for histopathologic examination. Two additional sections were taken from the upper and lower jejunum and frozen in sterile glass tubes to demonstrate the presence of TGE virus by immunofluorescence. The time interval between euthanasia and complete removal of all sections did not exceed 20 minutes.

Technique for Scanning Electron Microscopy

Fixation. The mucosal surface was exposed by cutting along the mesenteric border and fastening each section onto a styrofoam block with 6 to 8 stainless steel pins. The sections were gently washed with 0.85% saline solution then fixed for at least 4 hours in 4.0% glutaraldehyde[j] buffered with 0.1 M phosphate and adjusted to a pH of 7.4 (Sabatini *et al.*, 1963). They were then washed and stored in Sorensen's phosphate buffer solution (pH 7.4) (Henry, 1964). The buffer was changed every 1/2 hour for the first 3 hours. All fixation, washing, and storage was at 2 to 6 C. All sections from Litter 2 were post-fixed in 0.5% aqueous osmium tetroxide[k] for 1 hour then washed with deionized distilled water before drying.

Freeze Drying. The freeze drying apparatus[l] was used to dry all the tissues from Litter 1 and 10 tissues from Litter 2. Each tissue block was submerged for 1-1/2 minutes in isopentate[m] cooled with liquid

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[j]Fisher Chemical Co., Detroit, Mich.

[k]Osmic Acid, Scientific and Industrial Chem. Co., New York, N.Y.

[l]Model 10-010, The Virtis Co., Gardiner, N.Y.

[m]Eastman Organic Chemicals, Rochester, N.Y.

nitrogen, then transferred into a precooled Pyrex desiccator placed inside a freezer. The tissues were freeze dried at -30 C. for 48 hours. The operating condenser temperature was maintained at -68 C. by use of liquid nitrogen, and the vacuum was 5 μ Hg. Following drying, the desiccator and tissues were removed from the freezer and slowly warmed to room temperature under vacuum. The vacuum was then slowly released, and the tissues were transferred to a storage desiccator with anhydrous calcium sulfate.[n]

Critical Point Drying. A commercial critical point drying apparatus (CPDA)[o] was used to dry 18 sections from Litter 2 and the CPDA of Adams[p] was used to dry all sections from Litter 3. Disc-shaped sections of intestine, approximately 1.5 cm. in diameter, were cut and attached to roughened aluminum discs with 10.0% gelatin.[e] Sections from Litter 2 were then placed for 15 minutes each in 25, 40, 60, 80, 90, 95, 100, 100% ethanol, 2 parts ethanol to 1 part amyl acetate,[q] 1 part ethanol to 1 part amyl acetate, 1 part ethanol to 2 parts amyl acetate, and 100% amyl acetate. They were then stored in 100% amyl acetate. Sections from Litter 3 were placed for 1 hour in each of the above mixtures. During this time, the sections in the fluids were cooled in an ice bath and agitated on a mechanical shaker[r] adjusted to slow speed.

The 18 sections from Litter 2 were placed into a cylindrical wire basket, separated by small criss-crossed wires, and inserted in the

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[n]W. A. Hammond Drierite Co., Zenia, Ohio.

[o]DCB-1, Denton Vacuum Inc., Cherry Hill, N.J.

[p]Dr. D. R. Adams, Dept. of Anatomy, Mich. State Univ., East Lansing, Mich.

[q]Mallinckrodt Chemical Works, New York, N.Y.

[r]Eberbach Corp., Ann Arbor, Mich.

drying chamber of the CPDA. The top of the drying chamber was sealed and the procedure of Anderson (1951) for critical point drying was followed. Seven sections were dried at a time and each batch was dried twice. Syphon cylinders of bone-dry carbon dioxide (CO_2) were used throughout.

Similar procedures were followed for critical point drying the sections from Litter 3 with some modifications regarding the time of liquid CO_2 exchange. The chamber inlet valve was opened and liquid CO_2 was introduced into the drying chamber. The chamber vent valve (CVV) was opened slightly to allow a slow, even flow of vaporized CO_2 to escape for 15 minutes and then closed for 15 minutes. The CVV was alternately opened and closed for 15-minute intervals and the pressure was maintained between 750 and 1,030 p.s.i. Each time the CVV was opened, clean paper toweling was placed beneath the outlet nozzle, and the first effluent was deposited onto the paper. The paper was examined for amyl acetate odor. This procedure was repeated for 3 hours, or approximately twice the time required for the amyl acetate odor to disappear. The chamber vent and inlet valves were then closed and the drying chamber was placed in a water bath at 48 to 50 C. The pressure increased and stabilized at approximately 2,500 p.s.i. and was maintained at that level for 10 minutes. The CVV was then slowly opened and all the vaporized CO_2 in the drying chamber was released over a period of 15 minutes. The drying chamber remained in the hot water bath until venting was completed. A maximum of 8 tissues were dried at a time then transferred and stored in a desiccator with anhydrous calcium sulfate.

Mounting and Coating. Disc-shaped and cross sections of freeze dried tissue from Litters 1 and 2 were cut with a sharp razor blade and glued[s] to round glass cover slips (12 mm.).[t] These were glued to aluminum stubs (14 mm.)[u] and extra glue was placed along the tissue edges for better conductivity. The tissues mounted on the aluminum discs from Litters 2 and 3 were glued to the aluminum stubs in a similar manner. The sections were vapor-coated with 50 to 100 Å carbon[v] and 200 Å gold-palladium[v] in an evaporator[v] equipped with a tilting, variable-speed rotary coater.

Tissue Scanning. Five sections were mounted at a time onto the viewing stage of the SEM.[w] The working distance ranged from 25 to 35 mm., specimen tilt varied from 0 to 55°, the magnification factor ranged from x 100 to x 6,825, and the accelerating voltages used were 11 and 21 KV. Black and white pictures[x] were taken of representative sections.

Technique for Histopathology

Sections were fixed in 10% formalin-sodium acetate solution and stained with hematoxylin and eosin according to established procedures (Luna, 1968).

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[s]Tube Koat, G. C. Electronics Div., Hydrometals, Inc., Rockford, Ill.

[t]Arthur H. Thomas Co., Philadelphia, Pa.

[u]Ernest F. Fullam, Inc., Schenectady, N.Y.

[v]Ladd Research Industries, Inc., Burlington, Vt.

[w]Model AMR-900, American Metals Research Corp., Burlington, Mass.

[x]Polaroid 4X5 Land Film, Type 55P/N, Polaroid Corp., Cambridge, Mass.

Immunofluorescence

Preparation of Full-Thickness Frozen Sections. Sections were stored at -70 C. They were slowly thawed to room temperature, mounted onto stubs and quick-frozen at -25 C. in a cryostat.[y] Eight-micron cross sections were cut and mounted on glass slides. They were covered with 100% acetone for 10 minutes at room temperature then overlain with hyper-immune TGE antiglobulin which had been conjugated with fluorescein isothiocyanate (Black, 1971) and incubated for 30 minutes in a 37 C. water bath. The conjugate was removed by two, 5-minute washes with phosphate-buffered saline (PBS) at pH 7.5. The slides were then dipped in distilled water to remove excess PBS. The excess water was drained and a coverslip was applied with 1:1 PBS and glycerol.

The mounted sections were viewed on a Zeiss FA[z] microscope using 12.5X eyepiece lenses, 16X and 40X objectives, BG 12 and UG 2 exciter filters, and OG 4 and GG 4 barrier filters.

Preparation of Intestinal Smears. Frozen intestinal sections were slowly thawed to room temperature, and material from the mucosal surface was scraped and smeared onto glass slides with a scalpel blade. The procedure of Black (1971), as described above, was followed in adding the TGE antiglobulin-fluorescein dye conjugate to the smears.

Pictures were taken of representative sections illustrating immunofluorescence.

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[y] International Equipment Co., Needham Heights, Mass.

[z] Matheson Scientific, Chicago, Ill.

RESULTS

One pig from Litter 1 and 2 pigs from Litter 2 died within 48 hours of birth. These pigs had little or no liquid diet in the digestive tract and the stomach and small intestine contained only mucus and gas.

Clinical Signs

The unexposed pigs maintained a normal appetite and remained active and alert throughout the experiment. The feces were light brown and had a creamy consistency.

Diarrhea was observed in exposed pigs as early as 15 hours after exposure (Table 3).

Table 3. Observation of diarrhea at the time of euthanasia in 3 litters of pigs with TGE

Litter No.	Observation at the Time of Euthanasia (hours)						
	6	12	15	18	24	36	48
1	NVS*	NVS	NA**	Diar.	Diar.	NA	NA
2	NVS	NVS	NA	Diar.***	NVS	NA	NA
3	NVS	NVS	Diar.***	NA	NVS	Diar.	Diar.

*No visible signs.

**Not applicable.

***Diarrhea present in 1 of the 2 pigs euthanatized.

The feces of exposed pigs changed from light brown and creamy to yellow and watery by 24 hours after exposure. Weakness, dehydration, and rough hair coat were also observed in pigs exposed more than 36 hours. The skin over the hindquarters of scouring pigs was often reddened and covered with liquid feces. There was no evidence of severe anorexia or vomiting in any of the exposed pigs.

Gross Lesions of the Digestive Tract

The unexposed pigs had normal appearing liquid diet curd in the stomach and normal ingesta in the intestine. The small intestine continued to contract for a time after death, forming circular folds. There was no evidence of gross lesions in any of the unexposed pigs.

Gross lesions were observed in the digestive tracts of all pigs 12 hours or more after exposure. The stomach was distended with a mixture of gas and semisolid, undigested, liquid diet curd. An increased amount of mucus was observed in the small intestine of pigs euthanatized 12 or 15 hours after exposure. There was very little contraction of the small intestine after death. There was some hyperemia of the gastric and mesenteric blood vessels and accumulation of cloudy, straw-colored fluid and gas in the lumen of the small intestine, particularly in pigs euthanatized 18 hours or more after exposure. The wall of the small intestine was more translucent and the subserosal blood vessels were more apparent in pigs exposed 18 hours or more. Those exposed pigs in Litter 2 which had no evidence of diarrhea had yellow, watery ingesta in the jejunum. There was also considerable distention of the posterior ileum and the colon with tan, pasty feces in the latter pigs.

Observations by the Scanning Electron and Light Microscopes

Unexposed Pigs. Examination of the small intestine by both scanning electron and light microscopy revealed long finger-like villi of varying lengths and configurations projecting from the basilar crypt area (Figures 1 and 2). Some villi were flattened at the tip or flared at the base and rounded in the middle (Figures 1 and 3). The surface of each villus was interrupted by irregular transverse and diagonal furrows of varying depth (Figures 1 through 3) and openings of numerous goblet cells (Figures 1 through 4). The cleft or ridge forming the extrusion zone at the tip of a villus was also seen (Figures 3 and 5). The surface of the villi in the lower jejunum and ileum was interrupted by many hemispherical projections (Figure 6). This was caused in part by the outward protrusion of the luminal surface of absorptive cells and may have been increased by vacuolation of the cytoplasm (Figure 5).

Exposed Pigs. No prominent or consistent microscopic intestinal lesions could be demonstrated through 12 hours after exposure. Early changes did occur by 15 hours as seen in Figures 7 and 8. There was evidence (Figure 7) of shortening of the villi, deposition of increased amounts of mucus on the surface, partial exposure of the circumvillar basins and crypt areas, and the transverse furrows appeared deeper and more irregular. Many of these same changes were also noted on histopathologic sections (Figure 8).

By 18 hours after exposure, there was more uniform and consistent atrophy of all villi in the upper and lower jejunum and the circumvillar basins and crypts were more exposed (Figures 9 and 10). The luminal surface of many epithelial cells was roughened (Figures 10 and 11). Some cells had separated from the lamina propria and were bulging into

Figure 1. Scanning electron micrograph of normal villi from the duodenum. Notice the varying lengths, sizes, and configurations of the villi. The luminal openings of goblet cells (A) and the transverse furrows (B) are prominent. x 130.

Figure 2. Photomicrograph of normal villi from the duodenum. The normal structures include a crypt of Lieberkühn (A), lamina propria (B), columnar epithelium (C), transverse furrow (D), and a goblet cell (E). H & E stain; x 135.

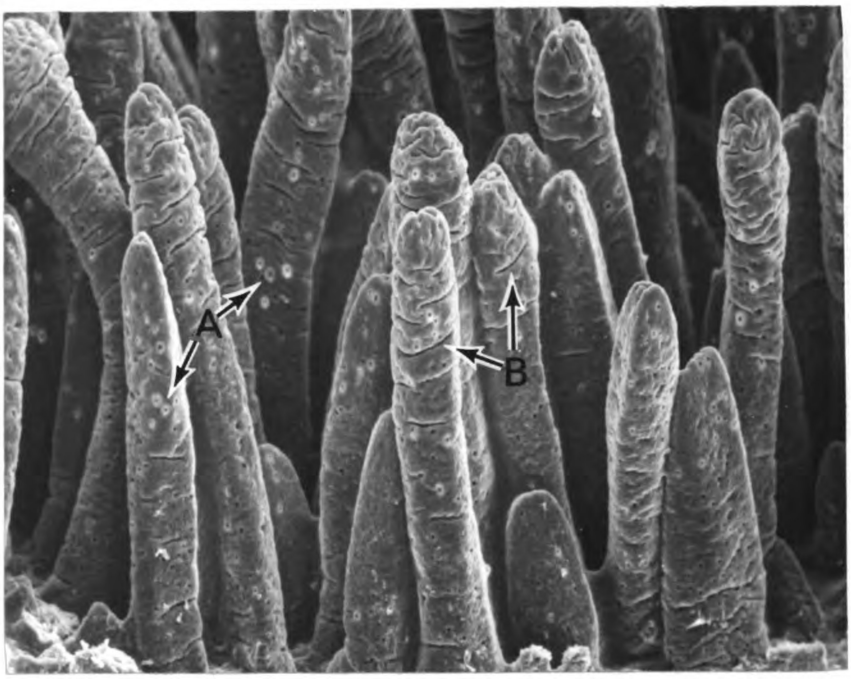

Figure 1

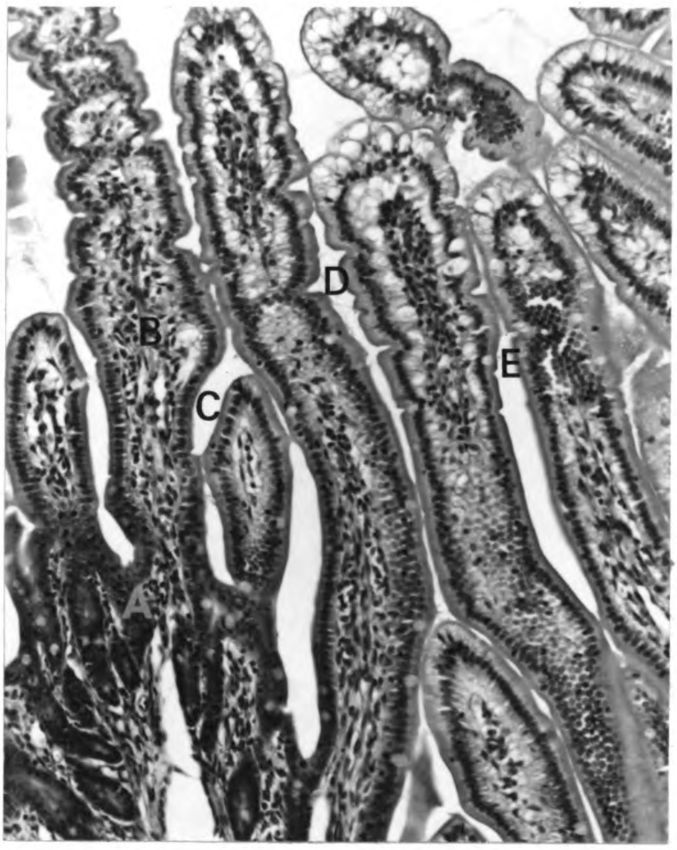

Figure 2

Figure 3. Scanning electron micrograph of normal villi from the duodenum. Notice the transverse furrows (A), flattening at the tip (B), openings of goblet cells (C), and an extrusion zone (D). x 320.

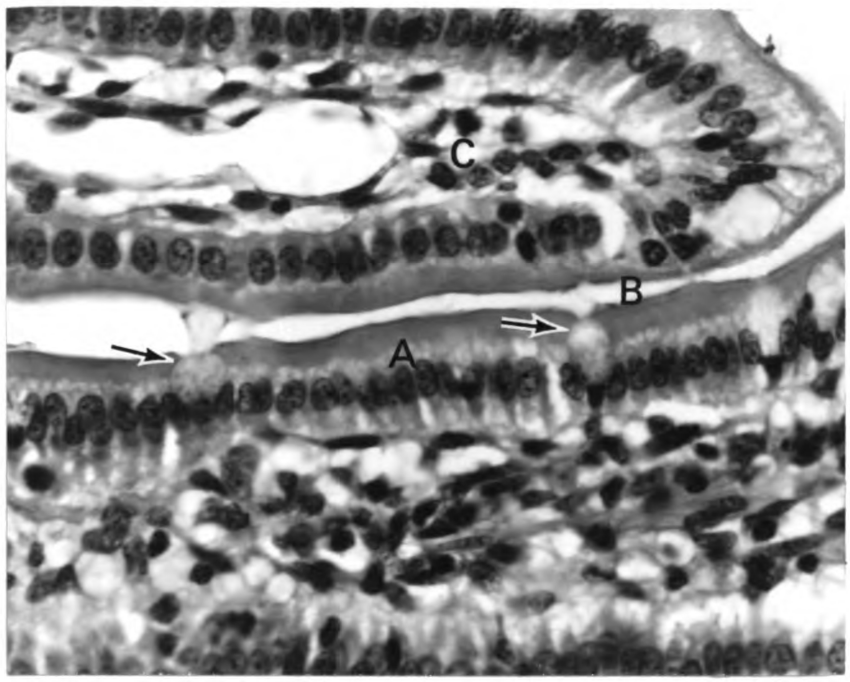

Figure 4. Photomicrograph of the luminal surface of 2 adjoining normal villi from the duodenum. Notice the columnar epithelium (A), brush border (B), lamina propria (C), and goblet cells which have partially discharged their mucous contents (arrows). H & E stain; x 540.

Figure 5. Photomicrograph of normal villi from the ileum. Notice the vacuolated cytoplasm and bulging of the luminal membrane of the epithelial cells (A). The arrow indicates an extrusion zone. H & E stain; x 135.

Figure 6. Scanning electron micrograph of the tips of normal intestinal villi from the lower jejunum. The luminal surface is interrupted by many hemispherical projections. x 697.

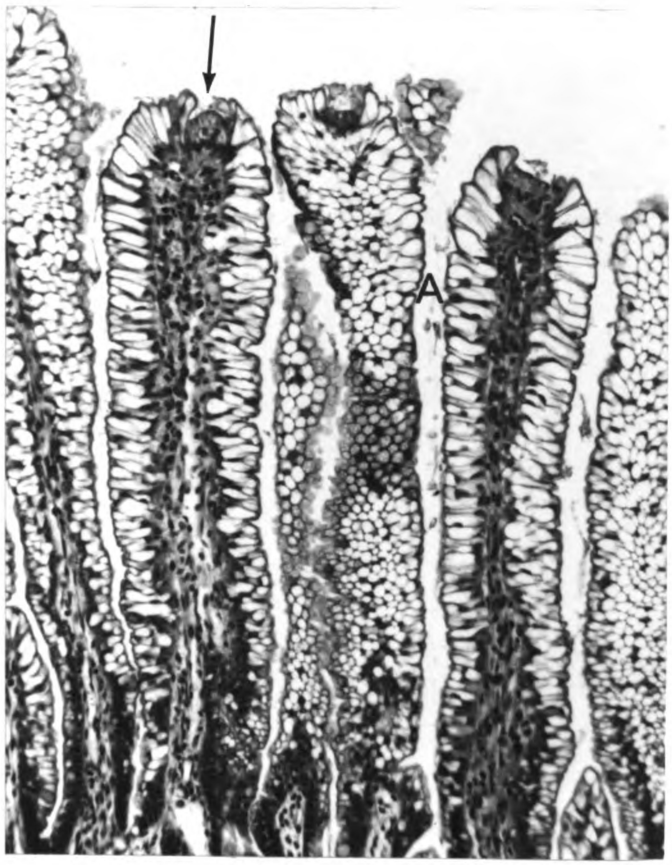

Figure 5

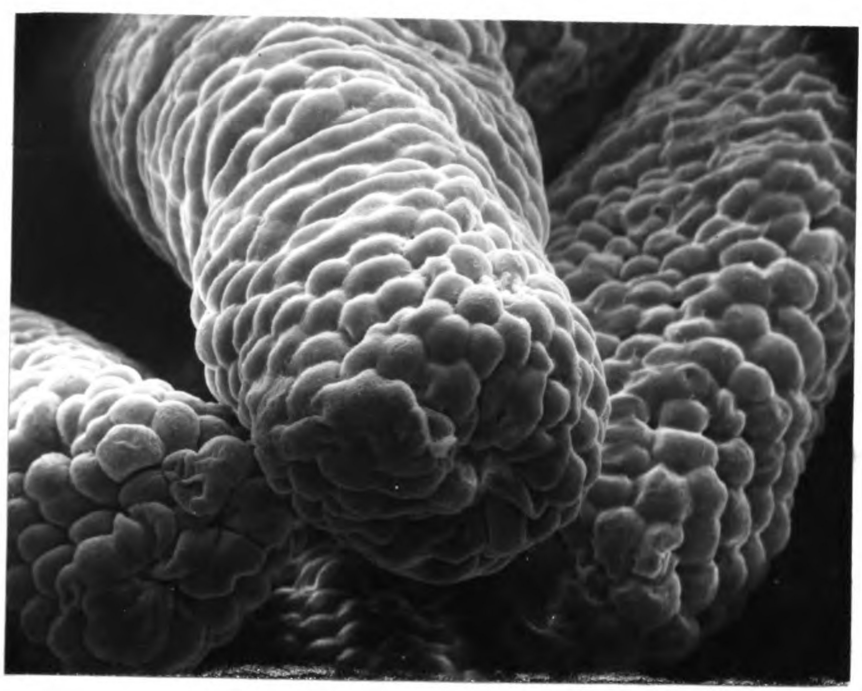

Figure 6

Figure 7. Scanning electron micrograph of the upper jejunum of a pig euthanatized 15 hours after exposure. The changes include shortening of the villi, partial exposure of the circumvillar basins (CV), irregularity and deepening of the transverse furrows and increased amounts of mucus (arrow). x 130.

Figure 8. Photomicrograph of the upper jejunum of a pig euthanatized 15 hours after exposure. Notice the shortened villi, the deep and irregular transverse furrows, dilated central lacteals (A), vacuolate epithelium (B), pyknotic nuclei (C), and indistinct brush border. H & E stain; x 135.

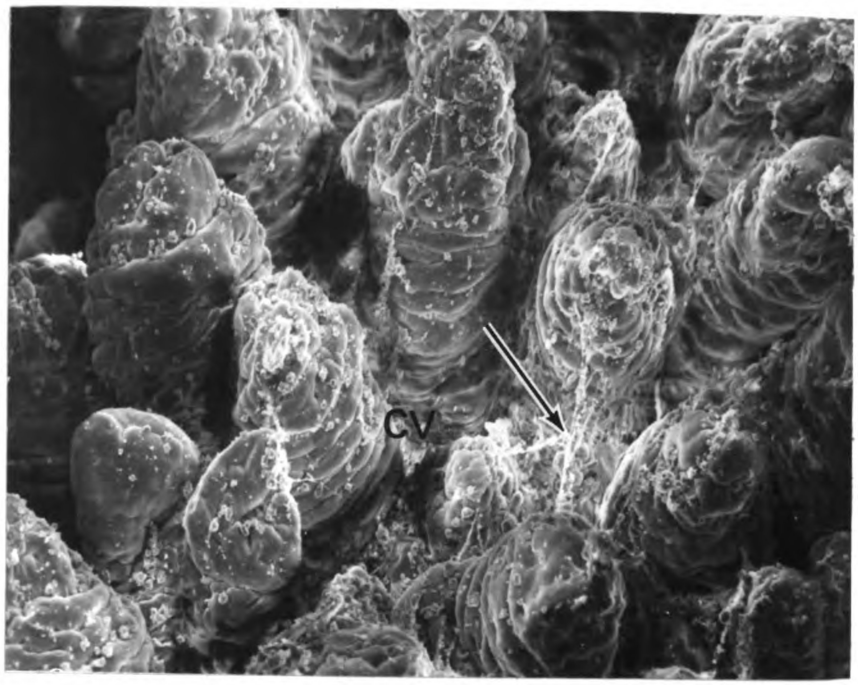

Figure 7

Figure 8

Figure 9. Scanning electron micrograph of the distal jejunum of a pig euthanatized 18 hours after exposure. The villi have undergone uniform atrophy and the circumvillar basins are exposed. x 120.

Figure 10. Scanning electron micrograph of 2 adjacent villi (18 hours after exposure) which have undergone extensive atrophy. Most surfaces of both villi appear to be covered by bulging and roughened epithelium, with the exception of the tips, where some of the epithelium appears to have sloughed (A). Notice the vestibules containing the openings of the crypts of Lieberkühn (B). x 685.

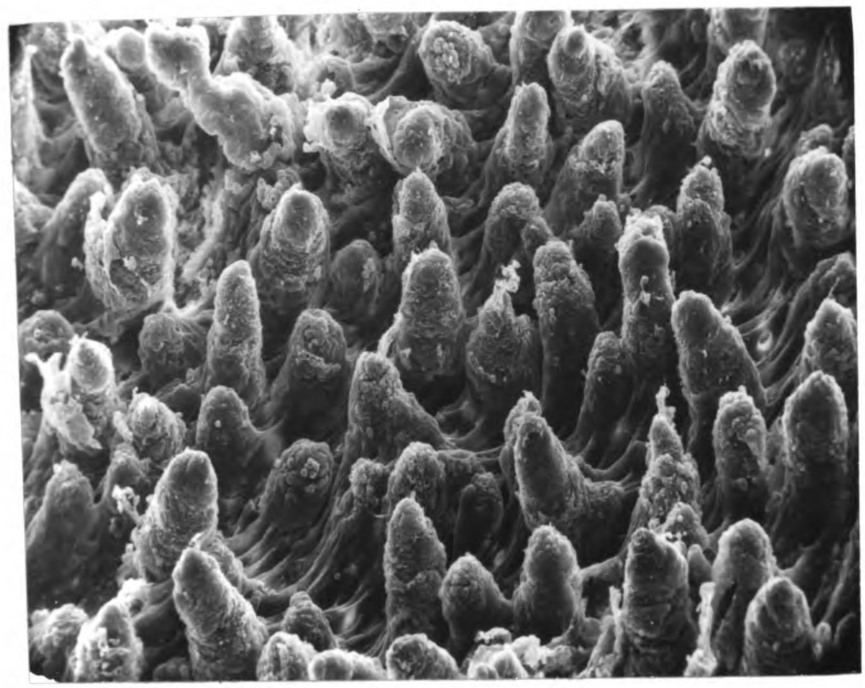

Figure 9

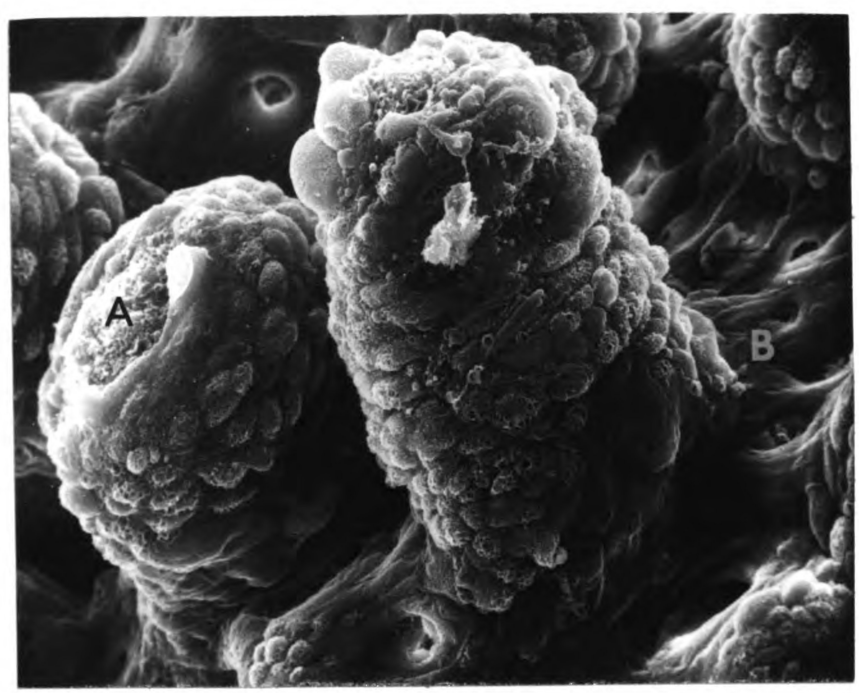

Figure 10

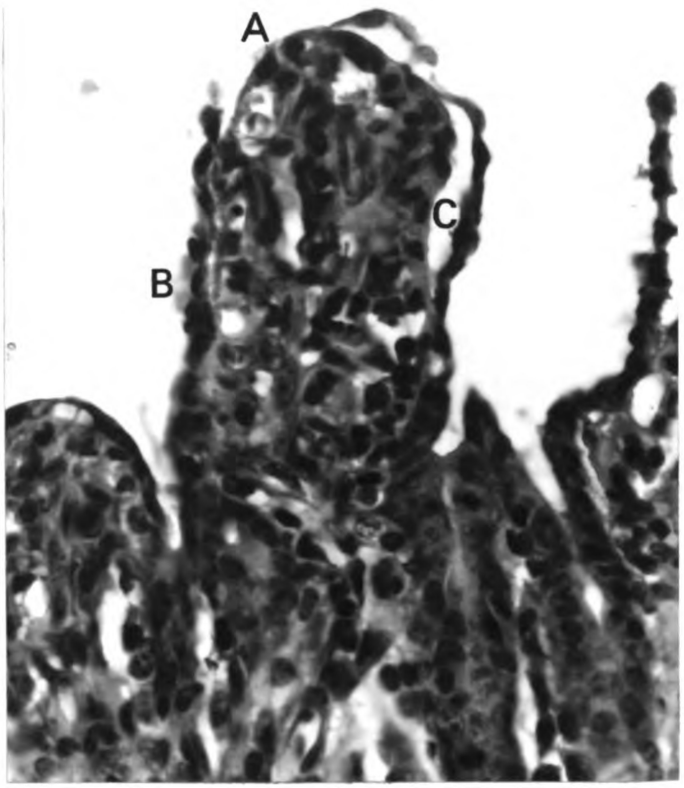

Figure 11. A photomicrograph of a single villus from the lower jejunum (18 hours after exposure) with metaplasia of the surface epithelium to low cuboidal and squamous cell types. Some cells have sloughed from the tip (A) and others appear roughened (B). Some have separated from the lamina propria and are bulging into the intestinal lumen (C). H & E stain; x 540.

the intestinal lumen (Figure 11). Metaplasia of the epithelium covering the villi was obvious on light microscopy (Figure 11) but was not apparent on scanning electron microscopy (Figure 10). Although most of the luminal surface of the villi was still covered by intact epithelium, individual and small groups of cells were separating and exposing the lamina propria (Figures 10 and 11).

Severe changes appeared in the jejunum and ileum by 24 hours after exposure (Figures 12 through 14). Some areas of the mucosal surface were almost bare, leaving only shrunken villi, exposed crypts and intervestibular ridges, and accumulations of mucus (Figures 12 and 13). Progressive atrophy often led to the coalescence of adjacent villi (Figure 13). Extensive bulging and sloughing of groups of epithelial cells from the tips and sides of villi were observed. The epithelial covering remained intact along the base of many villi, however (Figure 14).

At 36 hours after exposure, similar changes were observed in all levels of the small intestine.

Complete atrophy of many villi was evident on scanning electron microscopy at 48 hours after exposure (Figure 15), although this was not as obvious on histopathologic sections (Figure 16). The mucosal surface in many areas was bare except for a few remnants of villi (Figures 15 through 17) and mucous plugs (Figures 15 and 17). Some irregular projections from the surface of the intestine (Figure 15) probably represented single and coalesced adjacent villi which had accumulations of amorphous and proteinaceous material on the tips (Figure 16). Some remaining villi were covered by bulging epithelium (Figures 16 and 17) and scattered mucous plugs (Figure 17).

Figure 12. Scanning electron micrograph (line of vision perpendicular to the plane of the tissue) of the upper jejunum of a pig euthanatized 24 hours after exposure. Villus (A), mucous plug (B), vestibule and crypts (C), and intervestibular ridge (D).

Figure 13. Scanning electron micrograph of the upper jejunum (24 hours after exposure) with 2 villi in the center joined together and bent to one side. Mucous plug (A) and strand (B), intervestibular ridge (C), and crypt opening (D). x 325.

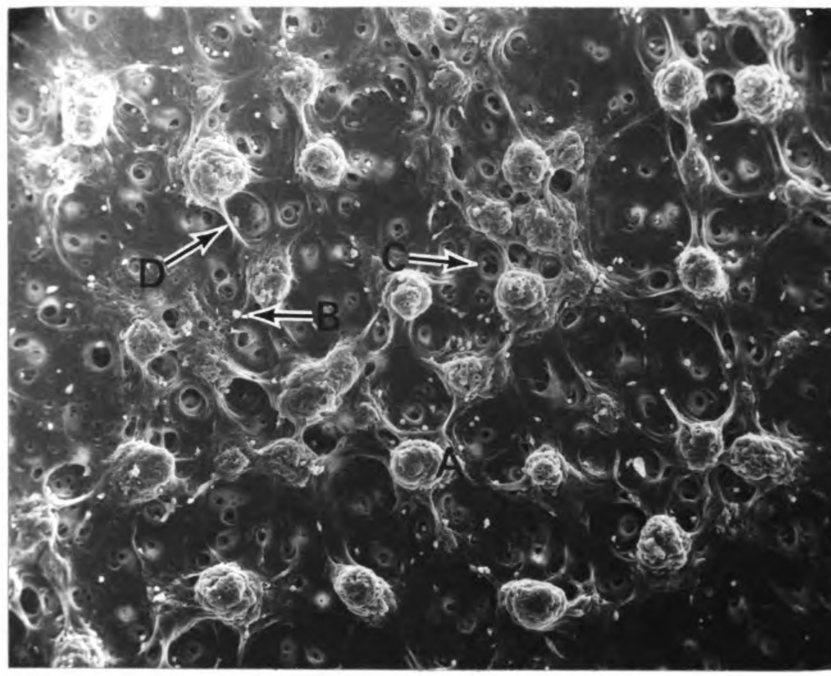

Figure 12

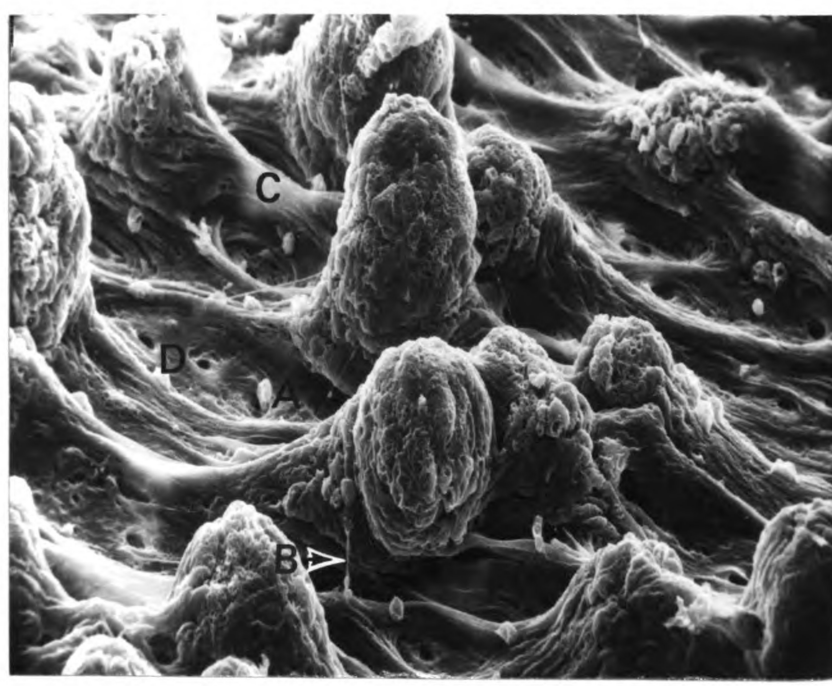

Figure 13

Figure 14. Scanning electron micrograph of the ileum (24 hours after exposure) with bulging and sloughing of large groups of epithelial cells (arrows). The lamina propria of the tips of the villi appears to be exposed whereas the epithelium is intact around the base of most of the villi. x 325.

Figure 15. Scanning electron micrograph of the lower jejunum of a pig euthanatized 48 hours after exposure. Notice the complete atrophy of some villi (A) and the raised remnants and projections of others (B). x 130.

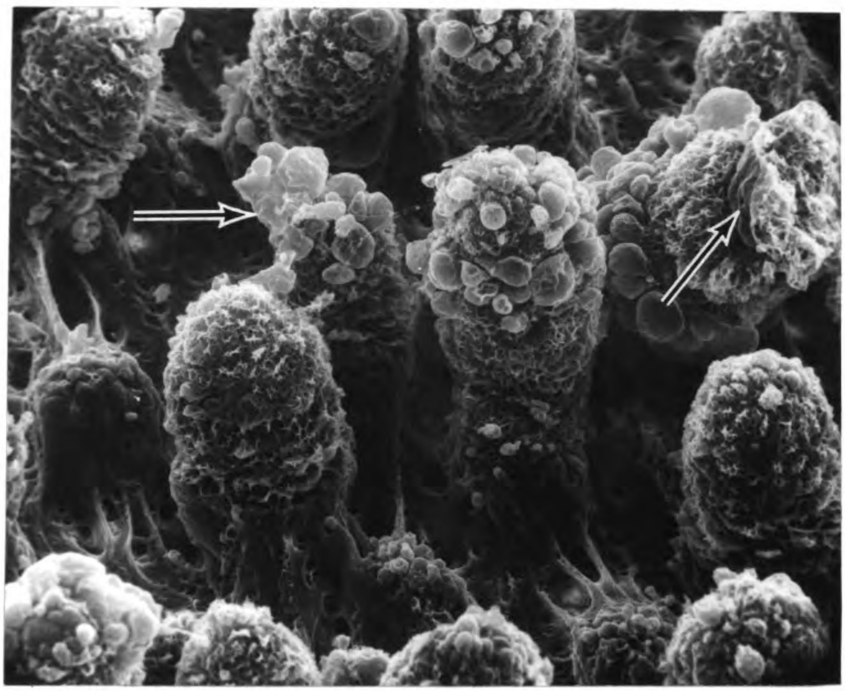

Figure 14

Figure 15

Figure 16. Photomicrograph of the lower jejunum (48 hours after exposure). Some villi have not undergone complete atrophy. Some are covered by degenerated and vacuolate cells (A) and amorphous proteinaceous material (B). H & E stain; x 135.

Figure 17. Scanning electron micrograph of a remnant of a villus from the upper jejunum (48 hours after exposure) which is covered by bulging epithelial cells (A) and mucous plugs (B), and surrounded by a series of vestibules filled with mucus (C). x 620.

Figure 16

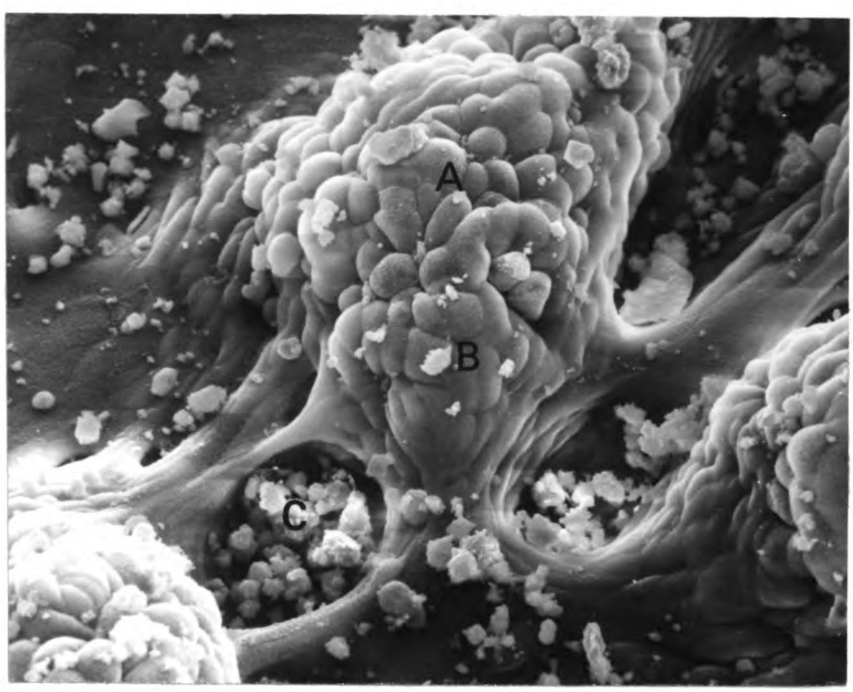

Figure 17

The proximal duodenum of pigs with TGE did not always escape damage to the villi (Figure 18), although the changes seen were not as extensive as those occurring in the jejunum and ileum. The microvilli on the surface epithelium of the crypts of Lieberkühn and vestibules (Figure 19) were apparently able to survive, although they appeared to be reduced in height and were indistinct. At 48 hours after exposure, microvilli were present on the surface of the epithelial cells of the jejunum (Figure 20).

The subgross and microscopic lesions of the small intestine in Litter 2 did not develop to the same extent as those observed in Litters 1 and 3. Villous atrophy and degeneration and sloughing of epithelial cells were not as obvious. Many villi were coalesced and lying to one side. Amorphous crystalline material was deposited on the tissues and the surface of many villi appeared wrinkled and distorted. None of the scanning electron micrographs or histopathologic photomicrographs from Litter 2 were used in the Results section. The reasons are covered in the Discussion section.

Immunofluorescence. All of the exposed intestinal sections from Litters 1 and 3 had some degree of positive immunofluorescence following addition of the hyperimmune TGE antiglobulin-fluorescein dye conjugate to the tissues (Figure 21). Only 2 of the exposed pigs from Litter 2 had positive immunofluorescence. None of the unexposed sections had any positive immunofluorescence following the same test.

Bacteriology. At the end of 3 weeks, all of the material for culture from the 3 litters tested was negative for the growth of bacteria or PPLO microorganisms.

Figure 18. Scanning electron micrograph of the duodenum (48 hours after exposure). Some of the villi appear to have undergone partial atrophy and there is some mucus on the surface. x 100.

Figure 19. Scanning electron micrograph of the duodenum (48 hours after exposure) with a crypt of Lieberkühn (A) filled with mucous plugs (B) and fine mucous strands (C). Note the granular surface which represents microvilli (D) and the linear outline of individual epithelial cells (E). x 1,600.

Figure 18

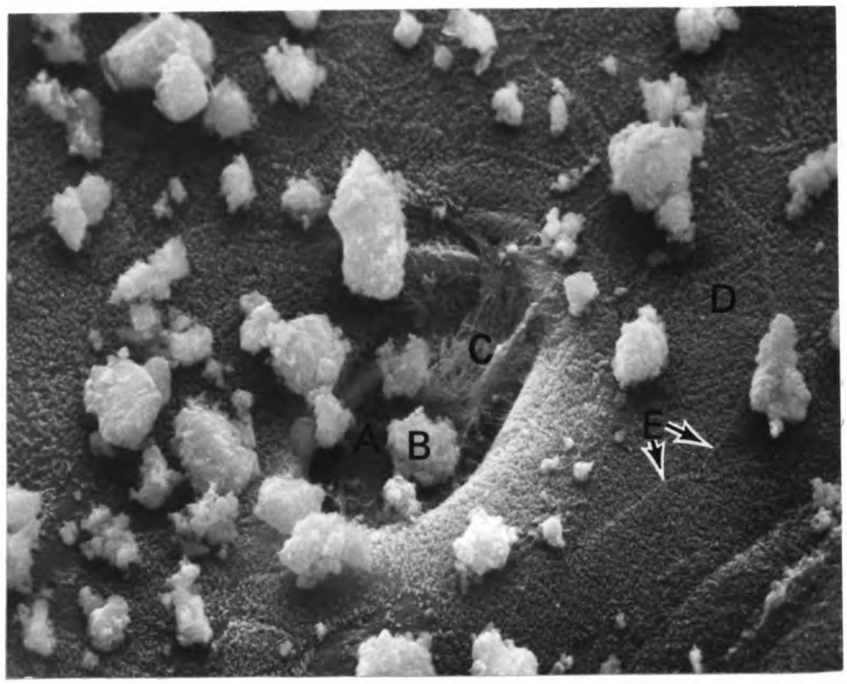

Figure 19

Figure 20. Scanning electron micrograph of the upper jejunum (48 hours after exposure). Notice the microvilli (A) projecting from the bulging surface of individual epithelial cells (B). x 6,825.

Figure 21. Photomicrograph of an intestinal smear from the upper jejunum of a pig euthanatized 24 hours after exposure. Notice the positive intracytoplasmic immunofluorescence (F) and the outline of the cell nucleus (A). The singular fluorescing bodies (B) probably represent nonspecific fluorescence. x 600.

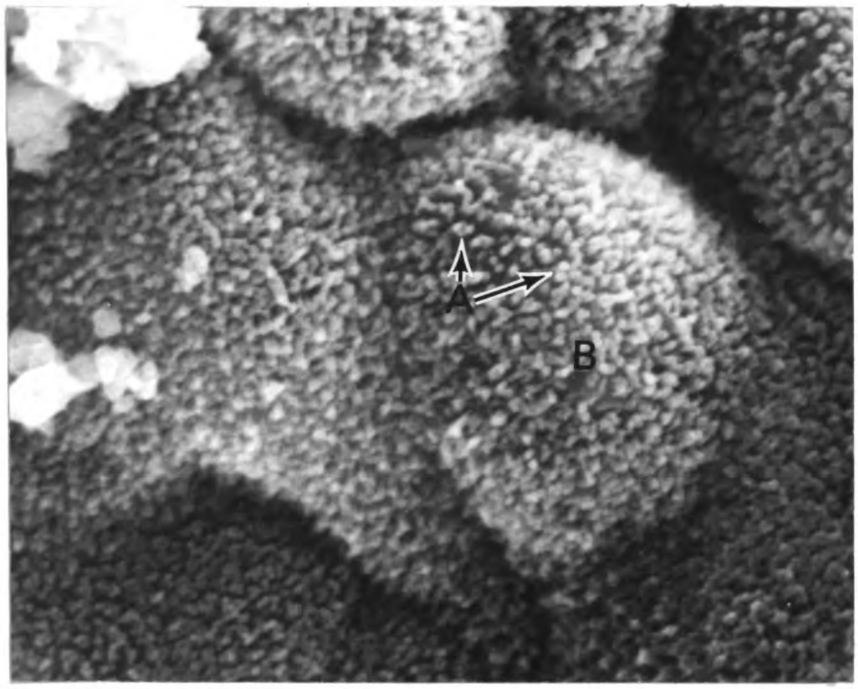

Figure 20

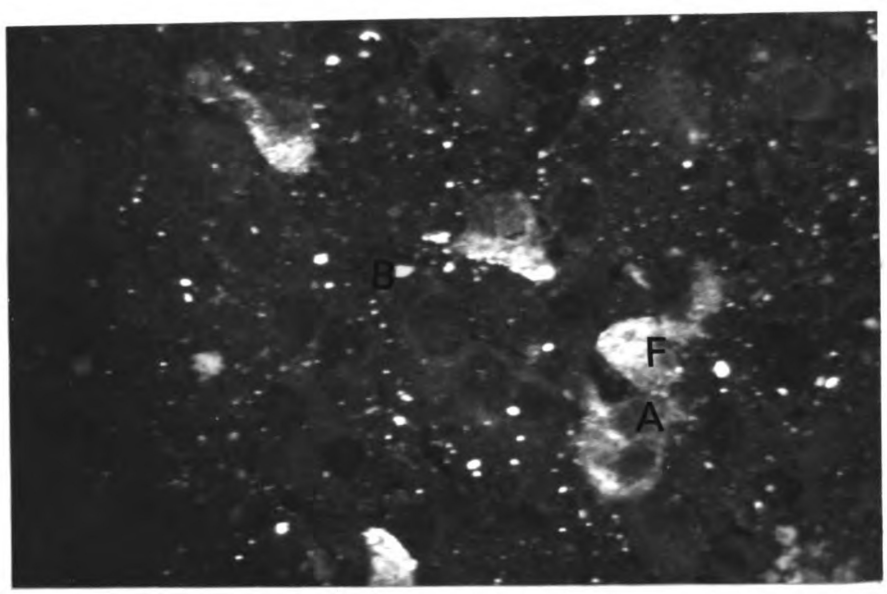

Figure 21

DISCUSSION

Many of the clinical signs and gross lesions observed in the 9- to 11-day-old gnotobiotic pigs (Litters 1 and 3) exposed to TGE virus in this study were consistent with those previously described (Bohl, 1970; Hooper and Haelterman, 1966a, 1969).

The exposed pigs in Litter 2 did not have clinical signs or gross and microscopic lesions at the various time intervals to the same degree as did the pigs from the other 2 litters. The expected gross and microscopic lesions were present but there was a delay of 6 to 12 hours in their development. The tissues from the pigs in this litter were generally unsatisfactory for observation with the SEM because of artifacts. The cause of these artifacts is unknown, but they may have been related to a failure to remove all the water from the tissues by the drying methods used. The inconsistent immunofluorescence of the exposed tissues would indicate that something may have happened in preparation or handling of the viral inoculum so that the pigs did not receive the calculated amount of virus.

The pigs from Litters 1 and 2 that died within 48 hours after birth were apparently unable to adjust to the shock of delivery by hysterotomy or acclimate to the environmental conditions of the rearing isolators or the liquid diet. There was no indication of any other underlying problem affecting these pigs other than a refusal to eat followed by rapid deterioration and death.

The feces of gnotobiotic pigs are normally creamy in consistency; therefore it was sometimes difficult to evaluate the early onset of diarrhea in some of the exposed pigs. Clinical diarrhea was noted in the present study earlier (15 hours after exposure) than that reported by Trapp *et al.* (1966). This occurred in spite of the fact that the pigs in the latter study received more viral inoculum (1×10^6 PID/ml.) than in the present study ($1 \times 10^{3.6}$ PID/ml.).

The apparent absence of vomition in the exposed pigs euthanatized 18 hours or more following exposure was noteworthy in view of the fact that this sign is mentioned so prominently in the literature. In many cases, the stomachs of exposed pigs were grossly distended with liquid diet curd and gas which seemed enough of a stimulus to incite nausea and vomiting. Possible explanations for the apparent absence of vomition include: (1) gnotobiotic pigs may not be as subject to vomiting following exposure to TGE virus as conventional pigs, (2) the liquid diet fed may have influenced the vomiting process, (3) the amount of virus inoculated into the pigs may have been insufficient to cause vomition, or (4) the examination of the pigs and the pans containing the waste material may not have been critical enough to detect the presence of vomitus.

Two procedures were used in this study in an attempt to remove all the ambient water from the fixed tissues and prepare them for scanning electron microscopy without causing any artifactual distortion. This was particularly challenging when dealing with full-thickness sections of intestine. There was evidence that all the water was not removed from the tissues following the freeze-drying procedures as outlined in the Materials and Methods section. The desiccator, used to hold the tissues, contained varying amounts of water even after 48 hours of freeze-drying. Apparently more time was needed to completely dry the tissues using this method. In scanning electron microscopy, a serious

consequence of residual moisture left in partially dried tissues is a phenomenon known as charging. Wet tissues will often absorb or reflect the high energy primary electrons and therefore will appear too dark or very bright, respectively. Electron flashes were also observed across the viewing screen. This interference leads to poor quality resolution and pictures. There was also evidence of charging, and the possible presence of residual water, in the tissues from unexposed pigs processed by the critical point drying method. A possible alternative to this procedure would be to place the tissues in 4 changes of 100% ethanol extending over a period of 12 to 24 hours. It was interesting to note that as the time between exposure and euthanasia increased, the intestinal sections appeared thinner. The thinner intestinal sections were easier to dry and there was less charging in these same tissues. Post-fixing the tissues in aqueous osmium tetroxide may increase the electrical conductivity of the tissues, reduce the amount of charging, and improve the quality of the scanning electron micrographs. Post-fixing the tissues from Litter 2, as outlined, did not reduce the amount of charging or improve the quality of the scanning electron micrographs in the present study. The suggestion was made (Spink, 1972) that better results might have been achieved if the concentration of aqueous osmium tetroxide had been increased to 2.0% and the fixing time increased to 4 or more hours.

The scanning electron micrographs in this study provided an excellent third dimension in surface visualization of the normal and subgross structures of the small intestine and lesions of TGE. The normal intestinal structures and the lesions observed by light microscopy correlated well with the structures and lesions observed by scanning electron microscopy. There are some limitations regarding the use of

scanning electron microscopy, however, which should be mentioned. Histopathologic sections of intestine are normally cut, stained, and viewed with the tubular intestine intact. The tissues prepared for scanning electron microscopy are laid flat. In certain instances, flattening of the tissues may create some artifact of the intestinal architecture. This may help explain why the circumvillar basins, particularly in pigs with TGE, were more apparent in scanning electron microscopy than in light microscopy. It is apparent that scanning electron microscopy of biological tissues, in most cases, should be coupled with examination of the same tissues by histopathologic techniques. For example, it was not possible to determine by scanning electron microscopy whether the hemispherical projections from the luminal surface of exposed and unexposed villi were due to vacuolation or desquamation of epithelial cells. Scanning electron microscopy only allows visualization of the surface structures,and an adequate description of normal and abnormal tissues requires an accurate viewing of both internal and external structures.

There was some variation in the extent of the subgross and microscopic lesions in exposed pigs euthanatized at the same time after exposure. This variation may have been a function of a slight difference in the amount of virus inoculated into the pigs or it may have been due to individual differences in response to the exposure.

Cytoplasmic vacuolation of absorptive epithelium of unexposed and exposed intestine has been reported in the literature (Alexander *et al.*, 1969; Christie, 1970; Drees and Waxler, 1970; Hooper and Haelterman, 1969; Moon, 1970). In the present study, vacuolation of epithelial cell cytoplasm also occurred in the lower jejunum and ileum but did not seem to alter the development of the microscopic lesions of TGE. The normal

histochemical activity of the absorptive cells is markedly altered in TGE (Cross and Bohl, 1969; Maronpot and Whitehair, 1967; Reber and Whitehair, 1955; Thake, 1968). This adversely affects the normal enzymatic and absorptive functions of the epithelium. Thake (1968) reported large fat globules within the cytoplasm of many exposed absorptive cells and suggested that these cells were incapable of transporting the fat to the lymphatic circulation. Thake (1968) also reported an alteration of the sodium transport process, leading to the suggestion (Haelterman, 1972) that the diarrhea of TGE is largely osmotic in character. The vacuolate cytoplasm of unexposed intestinal epithelium is probably due to the normal absorption processes, whereas some of the vacuoles in exposed cells may be due to degenerative processes.

The scanning electron micrographs and photomicrographs in the present study indicated extensive degeneration and desquamation of exposed epithelial cells from the surface of the villi which was in agreement with similar findings reported by Bay *et al.* (1951), Thake (1968) and Trapp *et al.* (1966). According to Haelterman (1972), desquamation was "in some instances preceded by disruption of the apical membrane and release of the cell contents into the lumen." Figures 10 and 11 (18 hours after exposure) are in agreement with this sequence of events and illustrate a disruption or roughening of the apical membrane of the exposed cells and early evidence of desquamation.

Hooper and Haelterman (1969) reported a maximum degree of atrophy of most villi within 1 day of exposing 1- to 2-week-old conventional pigs, kept in isolation, to TGE virus. In another study, Haelterman (1972) reported that villous atrophy may be complete in about 24 hours. Photomicrographs and scanning electron micrographs from the present study

indicate that maximum atrophy of most villi did not occur until at least 36 to 48 hours after exposure. Haelterman (1972) stated that the speed at which villous atrophy occurs may be dependent upon the quantity of infecting virus, age of the pig, virulence of the infecting virus and, probably, the presence of passively derived antibody in the lumen. This difference may also be a function of the different responses that gnotobiotic pigs may have to the TGE virus as compared to conventional pigs.

Trapp *et al.* (1966) reported that

> "Even though villi fused and seemed to close the crypt opening, glands and crypts were not cystic, indicating that at some point in the mucosa, access to the lumen remained open."

The scanning electron micrographs from the present study illustrate that the crypts did maintain access to the intestinal lumen through the network of vestibules in spite of atrophy and fusion of many villi.

The majority of the scanning electron micrographs and photomicrographs in the present study illustrate the progressive lesions of TGE. Replacement of desquamated cells with regenerated cells from the crypts may occur as early as 24 hours after exposure (Haelterman, 1972). The cells covering the villous stubs in Figure 17 (48 hours after exposure) in the present study may possibly represent regenerated cells which have migrated from the adjoining crypts. The structures on the surface of these cells may also represent regenerated microvilli (Figure 20).

SUMMARY

Twenty-nine gnotobiotic pigs from 3 crossbred gilts were utilized to study the progressive morphologic changes in the small intestine following oral exposure to transmissible gastroenteritis (TGE) virus at 9 days of age. Pigs were euthanatized at 6, 12, 15, 18, 24, 36 or 48 hours after exposure and comparisons were made between histologic lesions and scanning electron microscopic lesions and clinical signs.

Clinical signs and gross lesions typical of TGE were observed in the exposed pigs from 2 of the 3 litters. Gross lesions were observed in the digestive tracts of all pigs euthanatized 12 hours or more after exposure. Diarrhea was observed in pigs as early as 15 hours after exposure.

The normal intestinal structures and lesions observed by light microscopy correlated well with the structures and lesions observed by scanning electron microscopy. Early subgross and microscopic changes in the small intestinal villi and related structures were observed beginning at 15 hours after exposure. They included shortening of villi, deepening of the transverse furrows, and partial exposure of the circumvillar basins and crypts of Lieberkühn. There was uniform and consistent atrophy of all the villi in the jejunum and ileum by 18 hours. Metaplasia of the epithelium covering the villi was obvious by light microscopy but was not apparent by scanning electron microscopy. The epithelium became vacuolate and sloughed from the surface of many

villi so that almost total denudation had occurred by 36 hours. Complete atrophy of many villi and total exposure of the circumvillar basins were observed by 48 hours. Villous atrophy and damage to the surface epithelium appeared to be more extensive by scanning electron microscopy. There was some variation in the extent of the subgross and microscopic lesions in exposed pigs euthanatized at the same time after exposure.

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VITA

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David P. Olson was born in Rush City, Minnesota, on September 28, 1935. He attended Rush City Public School, Rush City, Minnesota, and entered the University of Minnesota in 1953. He was admitted to the Minnesota College of Veterinary Medicine in 1956 and graduated in 1960.

In June of the same year he joined a group practice in Worthington, Minnesota, and was engaged in a mixed practice until July, 1969. At that time, he accepted a full-time position as Instructor, and joined the staff in the Department of Large Animal Surgery and Medicine, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan. He was accepted as a graduate student in the Department of Pathology in 1969.

The author married Jane Marie Nieters of Cook, Minnesota, in June of 1959. They have 3 children, Dree Ann 11, Steven William 9, and Alane Marie 7.