ENTERIC COLIBACILLOSIS IN GNOTOBIOTIC SWINE: FLUORESCENCE AND ELECTRON MICROSCOPIC STUDIES

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D. L. Wagler
Major professor

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ABSTRACT

ENTERIC COLIBACILLOSIS IN GNOTOBIOTIC SWINE: FLUORESCENCE AND ELECTRON MICROSCOPIC STUDIES

By

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Four experiments using 40 gnotobiotic pigs were conducted to study the light and electron microscopic lesions produced by *E. coli* 0138:K81:NM. The pigs were exposed orally to approximately 3 million enteropathogenic *E. coli* organisms at 1 to 2 hours of age (early) or at 40 to 120 hours of age (late).

The organism was identified and localized in the tissues by the fluorescent antibody (FA) technique. Hyperimmune anti-*E. coli* serum, produced in rabbits, was labeled with fluoresceinisothiocyanate and used to stain the tissue sections.

It was found that the FA technique could be applied to paraffin-embedded tissues fixed in 95% alcohol, 10% buffered formalin or a mixture of absolute ethyl alcohol and acetic acid as well as to frozen tissues. The *E. coli* organism fluoresced a bright apple-green against a dull orange-red autofluorescence of the tissues. No diffuse

specific staining due to antigenic *E. coli* endotoxin or enterotoxin was observed.

The organism colonized the entire digestive tract. It was present in larger numbers in the stomach and duodenum of the early-infected pigs than in the late-infected pigs. The organism was commonly associated with the upper half of the villi and rarely populated the intestinal glands or the intervillus space at the base of the villi in the late-infected pigs. In the early-infected pigs which clinically were more severely affected, the organism was present in larger numbers at the base of the villi and in the intestinal glands.

It was concluded that the organism does not have to invade the intestinal epithelium to cause diarrhea. The organism did not invade the pharyngeal or gastric mucosa, rarely invaded the epithelium of the duodenum and invaded the epithelium in increasing frequency at more distal levels of the intestine. Invasion of the epithelium occurred only in the upper third of the villi and was much more frequent in the early-infected pigs than the late-infected pigs.

Light microscopic lesions of the intestine were mild. They were first noted at 10 hours after exposure and primarily consisted of edema of the lamina propria and dilatation of the central lacteal.

The initial ultrastructural changes in the ileal epithelium, present at 8 hours after exposure, were a decrease in pinocytotic vesicles, dilatation of the intercellular spaces, and accumulation of fat globules in the cytoplasm. As the disease progressed, there was an increase in number of cells showing regressive changes identical to the degenerative changes present in cells of the germfree controls undergoing necrobiosis.

The presence of organisms in immediate contact with the brush border did not visibly damage the microvilli. There was no evidence of attachment between the organisms and the microvilli. The organisms appeared to enter the cells one at a time and were always free within the cytoplasm. Bacteria were found only in cells with evidence of degenerative changes.

In the severely affected pigs, the posterior jejunum, ileum and large intestine were flaccid and remained in this condition until the pigs were killed, but in the less severely affected pigs the atony of the small intestine was temporary. In the pigs in which atony persisted, the organisms colonized at the base of the villi and in the intestinal glands. There was also an increase in epithelial intercellular spaces along with edema of the lamina propria. It is proposed that these changes were associated with hypomotility of the small intestine.

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By

David T. Drees

A THESIS

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To my wife Janet and our children

David Michael

Elizabeth Ann

Mary Susan

Jane Ellen

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INTRODUCTION

Diarrheal disease constitutes one of the leading causes of mortality and morbidity of swine throughout the world. The role of *Escherichia coli* as a causative agent of diarrhea has been disputed for over 70 years. The ubiquitous nature of the *E. coli* organism has contributed to this confusion. The same strains of *E. coli* that are present within the gut of diseased animals are also present in healthy animals. Only with the development of reliable methods for identifying the numerous strains of *E. coli* did the confusing picture begin to clear. Today the causative role of *E. coli* in diarrheal diseases of man and animals is well established.

The recent development of germfree or gnotobiotic techniques for rearing swine has been of major importance in the study of colibacillosis. These techniques have made possible the study of a single pathogenic strain of the organism without commensal organisms and antibodies clouding the study of the disease syndrome.

The objectives of this study were:

1. To develop a reliable technique to localize and identify the *E. coli* organisms within the tissues of experimentally infected gnotobiotic swine.

2. To determine if the presence or concentration of the organisms in tissues are associated with the specific lesions of the disease.

3. To determine the tissues most susceptible to invasion by the organism.

4. To determine if *E. coli* 0138:K81:NM produces the same ultrastructural lesions in the ileal epithelium of young swine as do other serotypes of *E. coli*.

LITERATURE REVIEW

Colibacillosis is a term used to indicate a variety of disease syndromes resulting from infection with certain strains of *Escherichia coli*. It originally applied only to infections of neonatal animals but today, because of the increase in knowledge, it is applied to disease syndromes of older animals as well (Jubb and Kennedy, 1963). The term "enteric colibacillosis" was used by Moon *et al.* (1968) to indicate the diarrheal disease syndromes in young swine caused by *E. coli*. Enteric colibacillosis has recently been divided into 3 disease syndromes: neonatal colibacillary diarrhea, weanling colibacillary diarrhea and edema disease (Nielsen *et al.*, 1968). The knowledge of *E. coli* infections, particularly *E. coli* infections of newborn animals, is rapidly expanding. It has been the subject of 3 recent reviews (Sojka, 1965; Barnum *et al.*,1967; Nielsen *et al.*, 1968).

The *E. coli* Organism

Escherichia is a genus of short, rod-shaped, gram-negative, nonsporulating bacteria belonging to the family Enterobacteriaceae (Breed *et al.*, 1957). These organisms are usually motile with long flagella and fimbriae, but

some strains are nonmotile and do not possess appendages (Barnum *et al.*, 1967). They are widely distributed in nature and are common inhabitants of the intestinal tract of man and animals (Merchant and Packer, 1961).

The species of organisms now known as *E. coli* was first isolated by Theobald Escherich in 1885 from feces of newborn breast-fed babies. He named the organism *Bacterium coli commune* and regarded it as a harmless saprophyte. Only four years later, in 1889, Laruelle implied *E. coli* might be a pathogen (Sojka, 1965). Since then, considerable effort has been directed toward characterizing the organism and investigating the contributing factors necessary to cause colibacillosis.

The *E. coli* group of organisms is composed of a number of different strains of bacteria and for over fifty years (1885-1943) biochemical methods of classification were the only means available to differentiate the strains. All attempts to biochemically differentiate between strains of *E. coli* cultured from infants with diarrhea, in which no other pathogenic organisms were present, and those from normal children were unsuccessful (Edwards and Ewing, 1962).

In 1943, Kauffman published the first of many articles describing serotyping of *E. coli*. His work explained the major obstacle which prevented the successful serological classification of *E. coli*--the O antigen

failed to agglutinate in specific O antisera because it was masked with a K antigen. After these antigenic characteristics were recognized, it became possible serologically to classify *E. coli* with accuracy. In 1947 Kauffman, utilizing his own results and those of Knipschildt and Vahlne, formulated an antigenic scheme for *E. coli* which is widely accepted today.

Since 1947 numerous additions have been made to this system, so that at present 147 O antigens, 89 K antigens and 49 H antigens are recognized (Barnum *et al.*, 1967). The K antigen can be divided into at least 3 varieties: L, A, and B. Detailed characteristics of *E. coli* antigens and procedures for typing the organism have been reviewed by Edwards and Ewing (1962), Sojka (1965), and Kauffman (1966).

Enteric Colibacillosis in Swine

Enteric colibacillosis has been studied for more than 70 years. During this time the investigational approaches to the problem can be divided into 4 different categories: (1) studies of naturally occurring outbreaks; (2) studies by experimental reproduction; (3) studies of intestinal loop infections; (4) studies utilizing *E. coli* enterotoxins.

Naturally Occurring Outbreaks

For many years the role of *E. coli* in enteric infections was disputed, even though as early as 1889 Jensen suggested that *E. coli* infections were associated with diarrhea in baby pigs. McBryde (1934) described an outbreak of diarrhea in neonatal pigs which he attributed to *E. coli* infection. He reported a high mortality in pigs 2 to 5 days of age and was able to isolate *E. coli* from many of the internal organs. Other investigators explained these isolations of *E. coli* from the infected pigs as the result rather than the cause of the diarrhea. They reached this conclusion because at that time it was impossible to differentiate between strains of *E. coli* isolated from diseased animals and those isolated from normal animals (Jubb and Kennedy, 1963).

Serological surveys of naturally occurring outbreaks of enteric colibacillosis have associated certain serotypes of *E. coli* with the disease. Bray (1945) was the first to make such an association in outbreaks of infantile diarrhea. The serotype he isolated is now designated 0111:B4, a well recognized enteropathogen in infants.

In 1958 Gordon and Luke described typical outbreaks of diarrhea in young pigs associated with *E. coli*. The pigs became depressed and anorectic and developed diarrhea. Their feces were profuse, watery and yellow to orange. The animals became dehydrated and comatose and

many died when only 24 to 48 hours old. Clinical signs of the disease appeared as early as 6 hours after birth but at times were delayed for a few days. They reported a high morbidity and mortality; often entire litters died. Postmortem examination revealed only a slight inflammation of the intestinal tract, and bacteriological examination resulted in the isolation of nearly pure cultures of *E. coli* from the stomach and small and large intestines. Occasionally the same strain of organism isolated from the gastrointestinal tract was also isolated from the liver and spleen.

Sojka *et al.* (1960) studied 2321 strains of *E. coli* isolated from various outbreaks of disease in swine. The most common serotypes of *E. coli* isolated were E57, E68 and G7. Classification of these organisms by the Kauffman-Knipshildt-Vahlne scheme identifies them as O138:K81(B), O141:K85a,b(B)K88a,b(L), and O8:K87(B?)K88a,b(L), respectively. Other important features of these isolations from the intestinal tract were the large numbers and purity of *E. coli* on primary culture. Saunders *et al.* (1960) further studied 58 of these outbreaks which occurred in pigs under 2 weeks of age. The 2 most common strains of *E. coli* isolated were O141 and O8. In the majority of the cases, only one strain predominated throughout the course of the disease and also in later recurrences in the same herd. In all cases the pigs were healthy and vigorous at

birth and "the significance of *E. coli* in these cases was obtained by a careful elimination of other possible causes." Lecce and Reep (1962) also reported *E. coli* 08 as enteropathogenic for swine.

Wittig (1966) investigated 83 fatal cases of *E. coli* infection in nursing pigs on 30 different farms. The *E. coli* isolated always contained K antigen 88L. This antigen was rarely identified in pigs of the same age that died of other diseases. The K antigen 88L was infrequently isolated in older animals infected with *E. coli*. He suggested that this may be due to the different diets of the two age groups.

In a study of 100 baby pigs, Gossling and Rhodes (1966) frequently identified 5 *E. coli* O antigens (08, 09, 020, 0101, and 0138) which tended to dominate the *E. coli* population in the intestine. They suggested *E. coli* serotypes 08:K85:H19 and 0138:K81:NM as possible primary etiological agents responsible for causing diarrhea in baby pigs. Moon *et al.* (1966a) associated 3 other *E. coli* serotypes (09:KV115(A), 08:KB5(B) and 0101:KV460(A) with enteric colibacillosis in young swine. Bacteremia was not a common finding, and morphological evidence of enteritis was absent.

A complete table listing all *E. coli* O groups and their association with disease in the various species of domestic animals was published by Barnum *et al.* (1967).

The different *E. coli* serotypes commonly associated with enteric colibacillosis in swine were tabulated by Nielsen *et al.* (1968).

Experimental Enteric Colibacillosis

Consistent reproduction of colibacillosis in conventionally reared swine has been unsuccessful. This difficulty creates the best argument against *E. coli* as the primary causitive agent of enteric colibacillosis in swine (Barnum *et al.*, 1967).

Only in recent years have successful reproductions of neonatal enteric colibacillosis been reported in conventionally raised pigs. Saunders *et al.* (1963a) were the first to report such a successful reproduction of colibacillosis. Twenty-one newborn pigs were exposed orally with 2 different serotypes of *E. coli* which had been isolated from typical field outbreaks of the disease. All the pigs developed diarrhea within 12 to 24 hours after exposure. Clinical signs and post-mortem lesions closely resembled those seen in natural outbreaks of the disease. Eleven of the 21 pigs died within 3 days. A profuse, pure culture indistinguishable from the inoculated strain of *E. coli* was isolated from the intestines and often from other organs. There was a close correlation between the isolation of the infecting organism from rectal swabs and the onset of diarrhea. In animals that clinically

recovered, the organism disappeared from the feces. No evidence of decreased susceptibility was shown by allowing the pigs colostrum before exposure to the organism. Although high dosages were used, equally large doses of a nonpathogenic *E. coli* did not produce diarrhea.

Using strains of *E. coli* isolated from field outbreaks of colibacillosis, Moon *et al.* (1968) were able to produce enteric colibacillosis in conventionally reared young pigs. In 20 pigs orally inoculated with *E. coli* 0101:KU460(A):NM, 11 developed enteric colibacillosis and 5 died within 6 days. Only 1 of 18 pigs inoculated with the enteropathogenic strain 08:K87,K88a,b:H19 developed diarrhea, and this animal died. The strain inoculated was recovered in almost pure culture from rectal swabs. It was not isolated from the visceral organs, other than the intestine, of those pigs that died and were necropsied. Inoculation with a nonpathogenic strain of *E. coli* did not cause disease.

Hysterectomy-derived or gnotobiotic pigs were especially susceptible to pathogenic strains of *E. coli*. Saunders *et al.* (1963b) were the first to report successful production of enteric colibacillosis in 9 pathogen-free pigs with as few as 1000 organisms per animal, using the oral route of exposure. Exposing young pigs to a pathogenic strain produced a disease syndrome similar to that which occurs in field outbreaks, but exposure of 3

pigs to a nonpathogenic strain resulted in diarrhea which soon subsided, and the pigs returned to normal. Two of these 3 pigs were then inoculated with the enteropathogenic strain and died 2 and 5 days later, respectively. The enteropathogenic strain was isolated in pure culture from the intestine, liver, heart, and brain. Since this report, a number of other investigators have described essentially the same experimental results in artificially raised swine. (Kohler and Bohl, 1966; Kohler, 1966; Kohler, 1967a; Kramer and Nderito, 1967; Staley *et al.*, 1969.)

In contrast to the commonly produced enteric syndrome, Britt and Waxler (1964) reported serofibrinous to fibrinopurulent polyserositis and polyarthritis as the principle lesions in gnotobiotic pigs exposed orally to *E. coli* serotype 083:K.:NM. Diarrhea and enteritis in these pigs were mild.

Christie (1967) was successful in producing heavy colonization of the intestinal tract, along with typical enteric colibacillosis, by injecting *E. coli* 0138:K81:NM into the stump of the umbilical cord of gnotobiotic swine. Extreme care was exercised to prevent oral exposure of these animals.

Intestinal Loop Infections

A test to study the enteropathogenicity of *Vibrio cholerae* was described by De and Chatterje (1953). This technique was later used to test and study the enteropathogenicity of various strains of *E. coli* isolated from swine. The organism to be tested is injected into a short segment of the small intestine which has been ligated at both ends. This prevents the movement of bacteria out of the small intestine due to intestinal motility. After 24 hours the pathogenicity of the organism is judged by the amount of fluid accumulated within the isolated loop of intestine. Using this technique, Moon *et al.* (1966b), Gyles and Barnum (1967), Smith and Halls (1967a) and Nielsen and Sautter (1968) reported similar results. Many strains of organisms isolated from field outbreaks of diarrhea and considered enteropathogenic produced distended gut loops while nonpathogenic strains did not cause fluid to accumulate. The numbers of organisms recovered from the positive and negative loops were similar. Therefore it was postulated that the enteropathogenic strains produce a toxin which causes a net flow of water into the lumen similar to that which occurs in enteric colibacillosis.

Smith and Halls (1967a) emphasized the importance of testing the suspected enteropathogenic organism in gut loops of the same species, since the results in different

species may not be uniform. For example, every strain isolated from pigs with enteric colibacillosis caused dilatation of ligated intestine in pigs, calves and lambs but not all of these strains caused the same results in rabbits. Ten strains associated with diarrhea in calves and lambs caused dilatation of both calf and lamb ligated intestinal loops, but none of these strains had any effect on ligated intestine of pigs and rabbits.

Enterotoxin Studies

As mentioned previously, the mere presence of large numbers of *E. coli* in the intestinal lumen was insufficient to produce diarrhea or distended ligated intestinal segments. Therefore, it has been postulated that certain strains must be able to produce a substance or substances toxic to the intestine. Recently, attempts have been made to characterize this toxic material, and the term enterotoxin has been applied to it (Smith and Halls, 1967b).

Smith and Halls (1967b) reported that bacteria-free fluids prepared from enteropathogenic strains of *E. coli* grown on soft agar produced distention when injected into ligated segments of the intestine. Distention was not produced by bacteria-free fluids prepared in the same manner from nonpathogenic *E. coli*. They presented evidence to indicate that this toxic material was not

endotoxin. The enterotoxins were present in larger amounts in the culture media than in the bacterial cells themselves. As little as 6 hours after inoculation of cultures, large amounts of enterotoxin were present in the medium. This is earlier than endotoxins are produced. The material disappeared from the medium earlier and was more heat-labile than endotoxin. Also, endotoxins prepared from the enterotoxin-containing cultures failed to produce distention of ligated intestinal loops.

Kohler (1968) administered bacteria-free filtrates of enteropathogenic strains of *E. coli* intragastrically to young pigs. In pigs 6 to 24 hours old, he reported consistent production of diarrhea in 1.5 to 3 hours after administration of the filtrates. The diarrhea lasted for 3 to 10 hours. Many pigs, 2 to 5 days of age, were resistant to the enterotoxin. He suggested that this increased resistance to enterotoxin may be related to increased physiological maturity which corresponds to the observation that naturally occurring colibacillosis occurs most frequently in pigs less than 7 days of age. Additional evidence was presented to differentiate the toxic material from endotoxin.

Pathogenesis

Currently, the majority of evidence indicates that *E. coli* organisms are ingested and move directly to the

intestine. In the intestine the organisms multiply, occasionally invade the intestinal epithelium and may enter the blood (Barnum *et al.*, 1967). Other investigators have proved that alternate routes of infection are possible.

To investigate the possibility of alternate routes of infection, Fey *et al.* (1962) prevented the direct movement of bacteria down the digestive tract in colostrum-deprived calves by ligation of the esophagus. They still were able to produce septicemia and colonization of the intestine by oral or intranasal inoculation. They concluded that infection of the intestine originated from the circulatory system rather than by direct movement down the digestive tract. Using gnotobiotic swine, Christie (1967) reported that *E. coli* injected into the umbilical stump can cause a bacteremia followed by colonization of the intestinal tract. The end result was a typical syndrome of enteric colibacillosis.

A large number of enteropathogenic *E. coli* in all regions of the gastrointestinal tract, particularly in the anterior small intestine, is nearly always associated with the disease (Kenworthy and Crabb, 1962; Smith and Jones, 1963). Smith and Halls (1967a), using the intestinal loop technique, reported the anterior part of the small intestine as the region most susceptible to dilatation by enteropathogenic strains of *E. coli*. Resistance to

dilatation increased in the posterior regions of the jejunum, and the ileum was often completely resistant to dilatation. They concluded that, to cause diarrhea, it is necessary for a strain of *E. coli* to proliferate in the anterior small intestine.

Many factors can contribute to increasing the number of *E. coli* in the anterior small intestine. A high level of environmental enteropathogens which are subsequently ingested can cause such an increase. Clinically, the current evidence suggests that, as the number of pathogenic organisms in the environment increases, the chances of infection in susceptible pigs also increases (Nielsen *et al.*, 1968).

Intestinal motility is important in controlling the number of organisms within the intestinal lumen. The movement of ingesta through the intestine by peristalsis is a natural mechanism to decrease the bacterial population in the anterior region of the small intestine. Any interference with this natural cleansing mechanism would increase the number of bacteria in the intestine (Dixon, 1960; Formal *et al.*, 1963).

The adhesive properties of enteric bacteria have been studied by Duguid and Gilles (1957). They concluded that the flagellate filamentous appendages on certain strains of *E. coli* are responsible for the adhesive properties. Therefore, certain strains of enteric bacteria with

appendages are able to colonize the intestinal lumen in high numbers. A similar conclusion was reached by Losos (1964). He observed large numbers of bacilli lining the free surface of the epithelial cells of the villi in *E. coli*-infected pigs and suggested the organisms were attached to the epithelium by fimbriae. In pigs affected with disease other than colibacillosis similar locations of bacilli were not observed. In an ultrastructural study of experimental colibacillosis, Staley *et al.* (1969) reported that fimbriae on *E. coli* (055:B5:H7) were first seen at 20 hours after exposure of the pigs. A high population of the organism was present earlier than 20 hours indicating fimbriae were not of prime importance in establishing an infection in the intestine with *E. coli*. They observed large numbers of the organism attached near goblet cells, suggesting mucin excreted by these cells as an important factor in attachment.

The neonatal piglet is most susceptible to enteric colibacillosis as reported both in field and laboratory studies (Dunne, 1964). This increased susceptibility of neonatal pigs may be explained by considering the pH of the stomach. The low pH in the stomach and anterior small intestine of older animals normally destroys or inhibits the growth of *E. coli*. The acidity of the stomach in the neonatal pig is not sufficiently high to inhibit proliferation of bacteria, so large numbers of *E. coli* are able

to pass into the anterior regions of the small intestine from the stomach, thereby increasing the susceptibility of the newborn pig (Smith and Jones, 1963).

Smith and Halls (1967a) suggested that the susceptibility of the newborn animal may be related to the physiological state of the intestinal epithelium. The epithelium of the small intestine in the very young animal may permit adhesion of enteropathogenic *E. coli* while in older animals this does not occur. Therefore, the peristalic movements which normally cleanse the anterior small intestine do not function efficiently. The number of bacteria increases, and the neonatal animal is more susceptible to enteric colibacillosis.

Lesions

Specific gross lesions have not been associated with neonatal colibacillosis. Pigs are usually dehydrated, their stomachs are filled with clotted milk and the intestines are congested, containing gaseous yellow material (Stevens, 1963; Dunne, 1964).

The observations of Kohler and Bohl (1966) suggest the period of time elapsed after death may influence the gross lesions present at necropsy. They reported no lesions in the small and large intestines of 8 gnotobiotic (*E. coli*-infected) pigs necropsied immediately after death. The mesenteric lymph nodes of 4 pigs were hyperemic. In

contrast, necropsies performed on 8 gnotobiotic (*E. coli*-infected) pigs, dead for 4 to 12 hours prior to necropsy, revealed lesions suggestive of enteritis in the jejunum and ileum. Congestion was common in many of the other abdominal organs.

Moon *et al.* (1966a) concluded that diarrhea occurs in *E. coli*-infected pigs without morphological evidence of enteritis. In contrast, Christie (1967) described a number of histological changes found predominantly in the gastrointestinal tract. The lesions varied from an acute hemorrhagic and necrotic enteritis to a histological picture consistent with that observed in normal germfree pigs. The most persistent change was hydropic degeneration in the epithelium of the villi. Subepithelial edema of the villi and neutrophilic infiltration of the lamina propria were also more commonly observed in the infected pigs than in controls.

Kohler (1967) observed a mild neutrophilic infiltration in the duodenum of pigs infected with enteropathogenic *E. coli*. He reported similar observations in pigs infected with nonenteropathogenic *E. coli* and suggested this reaction as the normal host defense response to the presence of bacteria. Microscopic examination under oil immersion (1000x) of Giemsa stained sections revealed small numbers of *E. coli* in the villi, submucosa and mesentery in all regions of the intestine. There was no

difference in location of the two strains of organisms in the intestinal wall, although only the enteropathogenic strain was isolated from the heart and liver.

Staley *et al.* (1969) also observed a neutrophilic infiltration of the lamina propria of the ileal villi along with a sporadic loss of microvilli at 20 hours after infection in neonatal piglets.

In a recent histochemical study Christie (1969) observed a substantial reduction of intracellular enzyme activity in the epithelial cells of the villi. The changes were most pronounced in the posterior regions of the small intestine. He concluded that *E. coli* enterotoxin produced a biochemical alteration in the epithelial cells which probably causes malabsorption, with diarrhea resulting.

In the past two years the electron microscope has been used to investigate the ultrastructural changes associated with *E. coli* infection in pigs. In 1967, Kenworthy *et al.* described the ultrastructural changes in the intestine of 5-week-old weaned pigs with diarrhea. The free surface of the jejunal epithelial cells was most severely affected. The entire plasma membrane became degenerate and separated from the terminal web, often resulting in the shedding of microvilli. The "blister" was filled with material of low electron density. In other cells the microvilli were lost or altered in length without

the formation of a "blister." The terminal web beneath the severely affected plasma membrane was disorganized or absent. Mitochondria were elongated and few in number. Degeneration of the lateral cell membranes allowed continuity of the cytoplasm between neighboring cells immediately above and below the desmosomes. Similar degenerative changes were observed in clinically healthy animals, and it was concluded that the changes in the infected animals differed only in that they were more severe.

Ultrastructural changes of the intestinal epithelium associated with experimental neonatal enteric colibacillosis in unfed, monocontaminated pigs, was described by Staley (1969) and Staley *et al.* (1969). Pigs were cesarian-derived, infected with *E. coli* 055:B5:H7 within 2 hours of birth, and killed 2, 6 or 20 hours later. Bacteria in the intestinal lumen prior to the 20-hour interval lacked fimbriae, and many degenerate bacilli were present at 6 hours. No cellular lesions or attachment of organisms were observed in the pigs killed at 2 or 6 hours after exposure. Twenty-hours after exposure, fimbriae had developed on the bacterial cell walls, and many organisms had attached to absorptive cells. In areas of attachment, often associated with goblet cells, microvilli were lost on 5 to 6 cells, and in neighboring cells varying degrees of distortion of the microvilli were observed. The terminal web beneath the areas of attachment was increased in

density, and the apical tubular system had degenerated. Bacterial invasion was observed only in the ileum. The bacteria entered the cell by invagination of the cell membrane, and once inside the cell, from 1 to 3 organisms were always surrounded by a single-layered, membrane-enclosed vacuole. Mitochondria and rough endoplasmic reticulum were numerous in areas of invasion. Many mitochondria were vacuolate, but vacuolate mitochondria were also observed in uninfected control pigs. Staley (1969) suggested that the microulceration may impair absorption of colostrum and other nutrients and allow fluids to be lost from the damaged cells, resulting in malabsorption and diarrhea.

Role of Colostrum

When discussing the susceptibility of the newborn animal to enteric colibacillosis, the role of antibody must be considered. Bauriedel *et al.* (1954) found no measurable gamma globulin in the serum of newborn, colostrum-deprived pigs. Therefore, if antibodies can exert a beneficial effect in the neonatal pig, they must come from colostrum. The results of many experiments designed to clarify the role of colostrum in the pathogenesis of enteric colibacillosis have not been consistent.

Owens *et al.* (1961) reported that orally administered colostrum, serum or gamma globulin were beneficial

in preventing colibacillosis. These same authors reported that parenteral administration of gamma globulin or swine serum had little or no value in preventing *E. coli* infections in colostrum-deprived pigs. Kohler and Bohl (1966) clinically observed a temporary beneficial effect following oral administration of specific immune serum to experimentally infected gnotobiotic pigs. Their results from *in vitro* studies indicated that beneficial effects occurred because of inactivation of endotoxin rather than lowering of the bacterial population in the intestinal lumen.

In a further study, Kohler (1967) clearly demonstrated that orally administered specific immune serum prevented diarrhea but did not affect the bacterial population within the intestine. A few bacteria were isolated from the liver and heart. Parenteral administration of serum also prevented diarrhea, but fluid loss from the intestine was greater than in the pigs in which serum was administered orally. No bacteria were isolated from the heart or liver. These results suggest that antibodies in the intestinal lumen have an antitoxic activity and that circulating antibodies may prevent the invasion of epithelium and the establishment of a bacteremia.

In studies utilizing the gut loop technique, Gyles and Barnum (1967) reported a reduction or absence of fluid accumulation in the ligated segment when specific antiserum

was injected along with enteropathogenic *E. coli*. In similar experiments, Smith and Halls (1967a) reported no decrease in fluid accumulated in the ligated intestinal segment.

The immunization of pregnant sows with enteropathogenic strains of *E. coli* in an attempt to increase the specific colostral antibodies and thus to protect the newborn pigs has also given contradictory results. Gordon and Luke (1958) reported that the use of autogenous *E. coli* bacterins administered to sows during pregnancy increased the resistance to *E. coli* infections in the offspring. In contrast, Jones *et al.* (1962), using autogenous bacterins and living organisms to immunize pregnant sows, reported no increased resistance in the baby pigs.

Smith and Halls (1967a) attempted to immunize older pigs with enterotoxin but reported no protection against the accumulation of fluid in subsequent gut loop studies. Kohler (1968) also attempted to immunize sows with enterotoxin so that the response of the nursing pigs to intragastric administration of enterotoxin could be studied. He reported no difference in response between the pigs born of vaccinated sows and that of pigs born to nonvaccinated sows.

The specific effect or effects colostrum plays in protection against enteric colibacillosis has not been clarified. Evidence indicates

that it may function within the intestinal lumen by neutralization of the toxin produced by *E. coli* and thereby prevent diarrhea. Circulating antibody appears to have limited value in preventing *E. coli* infections within the intestinal lumen, although it may increase the resistance of the epithelial lining to invasion and aid in the prevention of a bacteremia.

Fluorescent Antibody Studies

The fluorescent antibody (FA) technique was introduced in bacteriology by Coons *et al.* (1942). They were able to stain and visualize the pneumococcal polysaccharide in sections of tissue from infected mice. The technique is dependent on the fluorchrome-labeled specific antibody reacting with an antigen. This reaction is made visible by the fluorescence microscope. The techniques and results of FA have been reviewed in detail by Nairn (1962).

In *E. coli* investigations, the FA technique has been primarily used as a rapid method to identify enteropathogenic strains of *E. coli* in human fecal smears, particularly from children. Whitaker *et al.* (1958) were the first to report such a successful application of the technique. One hundred twenty-eight fecal specimens, collected during an outbreak of infantile diarrhea and stored for 3 years at -20C., were restudied by the FA

technique. Originally, 53 of the specimens were culturally positive due to the presence of *E. coli* 0127:B8. In addition to exact correlation with the 53 originally positive cases, 39 more were positive to the FA test. In contrast, at the time of the second study, *E. coli* could be isolated from only 24 of the original 53 positive cases. The increased number of FA-positive results in comparison with cultural methods was explained as being due to non-viable organisms, therefore indicating greater sensitivity of the FA technique.

Nelson and Whitaker (1960) used polyvalent labeled O antisera against 10 principle types of enteropathogenic human *E. coli* to study the specificity of the conjugates. They studied rectal swabs of 275 children under 2 years of age with signs of enteritis. The FA test gave more positive results for enteropathogenic *E. coli* than cultural methods; and on reculture of fluorescence-positive, culture-negative cases, a number of positives were found. Their results established the specificity of the FA test in that nonpathogenic *E. coli* and other bacteria commonly inhabiting the intestinal tract were not stained by the fluorescent-labeled antibody. They also pointed out that therapeutically administered antibiotics inhibited growth in cultures but did not interfere with the FA test.

The FA test was employed by Page and Stulberg (1961) to trace the spread of the infecting organism in hospitals. They were able to detect small numbers of organisms by direct staining of fecal smears and supported these findings by routine bacteriological studies.

The FA test for identifying Salmonellae in human fecal smears is not highly specific (Thomason *et al.*, 1959). Considerable cross staining occurs among the various serotypes of Salmonella, Shigella, Citrobacter and Arizona. To further evaluate the specificity of the FA test for *E. coli* Thomason *et al.* (1961) tested pure cultures of several Enterobacteriaceae and also fecal samples from patients with diarrhea. They recommended the use of OB antisera because OB-labeled globulin could be prepared with a higher titre than O globulin. Furthermore, the O antigens are less specific than B antigens, and fresh samples of *E. coli* may possess B antigens which can prevent staining by O-conjugated globulins. Their results suggested that the FA technique was equally specific for enteropathogenic *E. coli* as conventional serological methods. They concluded that the FA technique was more rapid than bacteriological and serological methods and could be an important tool in the early diagnosis of infantile diarrhea.

In a field study of 315 Puerto Rican children, Cherry *et al.* (1961) confirmed the conclusions of

Thomason *et al.* (1961). They emphasized that, in an outbreak of infantile diarrhea, representative FA-positive samples should be cultured and completely typed serologically to accurately identify the pathogenic *E. coli*. Therefore, FA-negative samples need not be serotyped. The prime usage of the FA test has been the screening of fecal smears and it provides a rapid and valuable tool in the management and treatment of infection due to *E. coli*.

The FA technique has been used to localize and identify viruses, bacteria, fungi, rickettsia and protozoa in the tissues of infected animals. The list of all the microorganisms, studied by this technique, is too long to be recorded here, but some of the bacteria genera studied by this method include: Pasteurella, Streptococcus, Staphlococcus, Salmonella, Bacillus, Listeria, Erysipelotrix and Klebsiella (Cherry *et al.*, 1960; White, 1961; Nairn, 1962). To the author's knowledge the Escherichia genus of organisms has not been studied in tissues by the FA technique.

MATERIALS AND METHODS

Animals

Forty germfree pigs derived from 4 sows by hysterotomy were used in this study. All were delivered on the 112th day of gestation except the exact breeding date of the dam of Litter 3 was not known. The general procedure, equipment, and techniques used for procuring and rearing gnotobiotic pigs as described by Waxler *et al.* (1966) and modified by Britt (1967) were followed. All the pigs were fed a sterile liquid-milk substitute* 3 times per day. During the first 24 hours after birth the pigs were given 3 ounces of milk per feeding. The volume of milk per feeding was increased to 4 ounces after the first day.

Each litter comprised one experimental group. The pigs from each litter were placed in 3 sterile isolators. Those in one isolator served as controls, while the remaining pigs in the other 2 isolators were exposed to the test organism.

The control pigs served to check the sterility of the intrauterine environment and the surgical procedure as well. They were killed at varying ages so that their

*SPF Lac, Borden Co., New York, N.Y.

composite ages at death would match the age range of the infected pigs at death.

The experimentally infected pigs were orally exposed to the test organism either early (1 to 2 hours of age) or late (40 to 120 hours of age). The pigs were killed at predetermined intervals ranging from 3 to 72 hours after exposure. The sex, age at time of exposure and age at time of necropsy for each pig are listed in Table 1.

Test Organism and Exposure of Pigs

The organism used in all experiments was *E. coli* 0138:K81:NM. It had been isolated from experimental pigs at Michigan State University and lyophilized in glass vials. Approximately 18 hours prior to anticipated exposure of the pigs, the lyophilized organism was inoculated into liquid thioglycolate medium and streaked on bovine blood agar plates. Both cultures were incubated at 37C, and the agar plates were observed for purity. Before the experimental animals were to be exposed the concentration of organisms in the thioglycolate medium was determined by McFarland nephelometer standards and diluted with sterile phosphate-buffered saline (sodium chloride solution) to approximately 3 million organisms per milliliter. Each experimentally infected pig was given 1 ml of this inoculum orally.

Table 1. Distribution of pigs, sex, age at exposure and age at necropsy.

Litter Number	Pig Number	Sex	Age at Exposure (hrs.)	Age at Necropsy (hrs.)	
1	0562	F	120	123	
	0563	M	120	126	
	0564	M	120	132	
	0565	M	120	144	
	0566	M	120	168	
	0567	M	120	192	
	0568	M	Control	176	
2	1567	F	1	5	
	1568	F	1	9	
	1569	M	1	13	
	1570	F	1	25	
	1574	M	40	50	
	1575	M	40	54	
	1576	F	40	58	
	1571	M	Control	10	
	1572	F	"	50	
	1573	F	"	52	
3	2401	M	2	6	
	2402	M	1	9	
	2403	F	1	13	
	2404	M	1	17	
	2405	F	2	26	
	2406	M	2	33	
	2407	M	2	34	
	2408	M	1	32	
	2409	M	2	30	(Dead)
	2410	M	Control	16	
	2411	F	"	35	
4	3655	F	1	9	
	3656	M	1	16	
	3657	F	1	25	
	3658	M	1	31	
	3659	F	1	39	
	3660	F	1	51	
	3661	F	60	76	
	3662	M	60	84	
	3663	F	60	96	
	3664	F	60	109	
	3665	M	Control	26	
	3666	M	"	98	

Determination of Bacteriological Status of Animals

The bacteriological status of the isolators and pigs was determined only twice because these were short term experiments. The first samples were collected soon after the pigs were placed within the isolators but before they were exposed to the test organism. Composite oral and rectal swabs were collected for culture. The second determination was made immediately before the last pig was removed from an isolator. For this determination swabs were taken from the mouth and from the waste collection pans in the bottom of each cage. Material from the swabs was streaked on bovine blood agar and MacConkey's agar plates and inoculated into semi-solid brain-heart infusion medium for anaerobic growth determination. The media were incubated at 37C and examined up to 7 days for evidence of bacterial growth. When bacterial growth occurred, smears were prepared from the brain-heart infusion tubes, stained with Gram's stain and examined by light microscopy. The methyl-red and Voges-Proskauer tests were used to determine bacterial species. The organism was also inoculated onto tryptose agar slants and stored at room temperature until samples from all 4 experiments were collected. All samples were then serotyped according to procedures described by Sojka (1965) to insure that the same serotype used to expose the piglets was also recovered following the experiment.

Necropsy Procedures

Immediately after removing the pigs from the isolators at predetermined times following oral inoculation (Table 1), the pigs were killed by stunning with a blow on the head and exsanguination. All necropsies were performed in an aseptic manner and were completed within 30 minutes after death to minimize multiplication and migration of the organism. One exception involved a pig (No. 2409) in Litter 3 which died unexpectedly and was not necropsied until approximately 4 hours after death.

A ventral midline incision was made with an electrocautery unit, and the skin covering the abdominal and thoracic cavities was reflected. The necropsy was continued with sterile instruments which were sterilized by flame before collection of each specimen. Specimens from the liver, lung, spleen, kidney, mesenteric lymph nodes draining the anterior and posterior portions of the intestinal tract and the mandibular lymph nodes were collected first. The intestinal tract was disturbed as little as possible during collection of these specimens. Specimens were then collected from the following areas of the digestive tract: pharyngeal tonsil; a 1-cm.-wide section of the stomach wall extending from the esophagus along the greater curvature to the pylorus so that all regions of the stomach were included; transverse sections of the duodenum; anterior, middle and posterior levels of the

jejunum; terminal ileum; cecum; and spiral colon.

To prevent contamination of the serosal surface by organisms flowing from the cut ends, ligatures were placed at both ends of the 1 inch long segments before the sections of small intestine were removed from the pigs in Litters 2, 3, and 4.

The specimens were placed in air-tight containers and frozen in liquid nitrogen (-120C) or fixed in 1 of 3 fixatives, 10% buffered formalin, 95% ethyl alcohol or a mixture of 19 parts absolute ethyl alcohol and 1 part glacial acetic acid. The fixed tissues were embedded in paraffin* and all tissues were processed for examination according to procedures described in the *Manual of Histologic and Special Staining Techniques*, Second Edition, of the Armed Forces Institute of Pathology, Washington, D.C. (1960). Serial sections were cut at 5 microns. One section of each specimen was stained with hematoxylin and eosin, one or more sections were stained with fluorescein isothiocyanate-labeled antiglobulin and other selected sections were stained with Giemsa's or Gram's stain.

Microbiological Studies

Specimens from the liver, spleen, lung and the mesenteric lymph nodes draining the midjejunum were

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*Paraplast Embedding Paraffin, Scientific Products, 1210 Leon Place, Evanston, Ill.

aseptically removed from pigs in Litters 3 and 4 for cultural examination. Approximately 1 gram of tissue was placed in Ten Broeck tissue grinders kept at OC. The tissue samples were ground with 5 ml. sterile saline. One milliliter from each suspension was pipetted into a Petri dish and mixed with 10 ml. melted MacConkey's agar. The agar plates were incubated for 18 to 36 hours and the bacterial colonies were counted.

Fluorescent Antibody Techniques

Preparation of K O Antiserum

Hyperimmune serum was produced by immunization of 5 to 6 lb. rabbits with 5 intravenous injections of whole *E. coli* 0138:K81:NM organisms which were grown in brain-heart infusion broth at 37C for 4 or 5 hours. The cultures used in the first 3 injections were formalinized by adding sufficient formalin to give a final concentration of 0.5%. The first formalinized broth culture was incubated at O C overnight and then injected into the rabbits. The second and third injections were made using cultures formalinized for only 2 or 3 hours. The last 2 injections were made with living cultures incubated for 4 or 5 hours. The first dose was 0.3 ml., second 0.5 ml., third 1.0 ml. and the fourth and fifth 2.0 ml. each. The injections were made at 4 or 5 day intervals and 50 ml. of blood were collected by cardiac

puncture 7 days after the final injection (Edwards and Ewing, 1962). The antisera were separated and tested by the tube agglutination method for "O" and "K" serum titres (Sojka, 1965). Sera with "O" titres of 1:1600 or higher and "K" titres of 1:100 or higher were pooled and stored at -20C until fractionation. Sera from normal (non-immune) rabbits were collected and stored in the same manner.

Fractionation and Conjugation of Globulins

The globulin fractions were obtained by 3 precipitations of the normal and hyperimmunized rabbit sera with 50% ammonium sulfate. The precipitated globulins were dissolved in distilled water and dialysed at 4C against 0.85% (Na Cl) solution. Frequent changes of the salt solution were utilized until it became free of ammonium sulfate. The globulins were conjugated with fluorescein isothiocyanate according to the ultra-rapid labeling technique of Rinderknecht (1962) which utilizes fluorescein-isothiocyanate dispersed on diatomaceous earth. The unconjugated dye was separated from the conjugated globulins by a gel filtration column of Sephadex G-25*. This method of labeling and removal of the free dye can be completed in 1 hour or less. The labeled globulins were preserved

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*Pharmacia Fine Chemicals, Inc., Rochester, Minn.

with merthiolate (1:10,000), separated into small aliquots and stored at -20C.

Staining Procedure

One volume Bacto FA Rhodamin counterstain* was added to 20 volumes fluorescein-conjugated globulin. The anti-*E. coli* conjugated globulin was diluted 1:10 with phosphate-buffered saline (pH 7.2). The normal globulin conjugate, used as a control, was not diluted. Both fixed tissues embedded in paraffin and fresh frozen tissues were stained by the FA technique. The paraffin-embedded tissues were sectioned, mounted on glass slides, deparaffinized with 2 changes of xylene, hydrated with a series of ethyl alcohols to 70%, passed into phosphate-buffered saline (pH 7.2), air-dried and layered with conjugate. The frozen tissues were sectioned with a cryostat, mounted on glass slides, air-dried and layered with conjugate. All layered sections were placed in a moist chamber and incubated at 37C. After 30 minutes incubation, the slides were washed in 2 changes of phosphate-buffered saline (pH 7.2) for 10 minutes each, mounted in buffered glycerin and covered with coverslips. Within 24 hours after staining, the tissues were examined on a standard microscope** equipped with a

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*Difco Laboratories, Detroit, Mich.

**Microstar, Series 10, American Optical Co., Buffalo, N.Y.

dark-field condenser and an ultraviolet light source using an Osram high-pressure mercury-vapor HBO-200 lamp. A Corning 5850 (4mm. thick) excitor filter and a Schott GG-1 barrier filter gave best results, although a Schott BG-12 excitor filter with a Schott GG-9 barrier filter also gave satisfactory results.

Photomicrographs were taken, using high speed color film,* with exposure times of 10 to 30 seconds depending upon the intensity of the fluorescence and autofluorescence of the section.

Electron Microscopic Techniques

During the necropsy procedure a 1-inch segment of the terminal ileum from the early infected pigs was removed and opened along its mesenteric border. The segment was flattened so that its serosal surface was adhered to a 2-inch-square area from an index card. This was submerged in 5% glutaraldehyde containing veronal actetate buffer (Palade, 1952) or phosphate buffer (0.15M)(Pitmann and Pitmann, 1966) for 3 hours. Some segments were placed in 2% glutaraldehyde containing the same buffers for 12 to 16 hours. The fixative was removed by washing the specimens in 4 or 5 changes of the respective buffer, the final wash lasting for at least 12 hours. The ileum was cut into

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*High Speed Ektachrome, EHB135, Eastman Kodak Co., Rochester, N.Y.

1 × 2 mm. pieces, postfixed in 1% osmium tetroxide in phosphate buffer for 1 hour and washed in 4 or 5 changes of the same buffer. The tissue was dehydrated in a graded series of alcohols and transferred to propylene oxide. After 2 baths of propylene oxide, 30 minutes each, the tissue was placed in a 1:1 mixture of propylene oxide and Epon 812 overnight (Luft, 1961). The following day, the specimens were embedded in Epon 812. The specimens were oriented in flat embedding molds* so the villi could be sectioned longitudinally. The Epon was cured at 25C for 6 hours followed by 60C for 36 hours. Silver to light gold sections were cut from the upper half of the villi with a diamond knife on a Porter-Blum M-2 ultramicrotome. The sections were mounted on carbon-coated 100-mesh copper grids or on uncoated 300- or 400-mesh copper grids. The sections were stained in uranyl acetate for 30 minutes and lead citrate for 15-25 minutes. Examination and micrographs were made with a Philips 100 electron microscope.

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*Ladd Research Industries, Inc., Burlington, Vt.

RESULTS

Clinical Observations

All of the pigs were active and alert soon after surgical delivery. The pigs in Litter 3 were small but otherwise appeared normal. Usually within 12 hours they were accustomed to eating from the feeding pans and consumed their liquid diet readily. Their feces were firm to pasty and varied from tan to brown.

All the pigs exposed to the *E. coli* organism, if allowed to live for 10 to 15 hours after exposure, developed clinical signs typical of enteric colibacillosis. The feces were watery, pale yellow to pale green, and usually contained small clots of undigested diet and bubbles of gas. The animals soon became dehydrated, although their appetites remained normal, except for 4 pigs (Nos. 2406, 2407, 2408, 2409) in Litter 3. Twenty-four hours after exposure these pigs were severely affected and partially anorectic. Their clinical condition continued to deteriorate until 1 died (No. 2409) at approximately 28 hours after exposure and the remaining 3 were killed and necropsied 31 to 32 hours after exposure.

Microbiologic Findings

Bacteriological Status of Animals

All samples collected for culture soon after birth were free of detectable microorganisms. The control pigs and their isolators remained free of organisms throughout the experiments. From all samples collected following exposure of the pigs, a pure culture of *E. coli* was isolated. These organisms were agglutinated by 0138 and K81 specific antisera, indicating that the same serotype of organism inoculated into the test pigs was also recovered at the conclusion of the experiment. Therefore, no mutation of the *E. coli* or contamination by other serotypes of *E. coli* had occurred.

Bacteriological Counts

In Litters 3 and 4, bacteriological counts were done on tissue samples from the kidney, liver, spleen and mesenteric lymph nodes to evaluate the number of live organisms in the visceral organs (Table 2). These counts were also used to check the accuracy of observing the *E. coli* organism in FA stained sections of these organs. Although the number of animals cultured was insufficient to give conclusive data, a tendency for higher numbers of organisms to exist in the visceral organs of the early-infected pigs as compared to the late-infected pigs was evident.

Table 2. Results of bacteriological counts.

Pig Number	Time of Exposure	Hours After Exposure	Kidney	Liver	Spleen	Mesenteric Lymph Node
2401	Early*	4	-	-	-	-
2402	"	8	-	-	-	-
2403	"	12	-	++	-	-
2404	"	16	-	+	++	+
2405	"	24	+++	+++	+++	+++
2406	"	31	+++	+++	+++	+++
2407	"	32	+++	+++	+++	+++
2408	"	31	+++	+++	+++	+++
2409	"	Dead	NC	NC	NC	NC
2410	Control	-	-	-	-	-
2411	"	-	-	-	-	-
3655	Early	8	-	-	-	-
3656	"	15	-	-	-	-
3657	"	24	+	-	-	-
3658	"	30	-	-	-	+
3659	"	38	-	-	+	-
3660	"	50	-	++	+	+++
3661	Late**	16	-	-	-	-
3662	"	24	-	-	-	-
3663	"	36	-	-	-	+
3664	"	48	-	-	-	-
3665	Control	-	-	-	-	-
3666	"	-	-	-	-	-

* 1-2 hours of age
** 60 hours of age
\- 0-2 organisms per plate
\+ 3-10 organisms per plate
++ 11-50 organisms per plate
+++ 51 or more organisms per plate
NC Not Cultured

Gross Lesions

No gross lesions were observed in the control pigs or in the 7 pigs killed within 8 hours after exposure. The intestinal contents were pale yellow and homogeneous. Pigs killed 10 hours or later after exposure consistently had diarrhea or impending diarrhea as evidenced by the presence of watery fluid in the colon and rectum. In pigs with clinical evidence of diarrhea, the watery fluid mixed with gas and small clots of undigested milk extended from the midjejunum to the rectum. The fluid and gas did not cause distention of the bowel. Pigs at the onset of diarrhea (10 to 15 hours after exposure) usually had a flaccid large intestine. After diarrhea persisted for approximately 2 hours (12 to 16 hours after exposure), atony of the intestinal wall extended forward to include the ileum and posterior half of the jejunum. The muscular tone of the small intestine partially, if not completely, returned by 30 hours after exposure except in the 4 severely affected pigs in Litter 3 (Nos. 2406, 2407, 2408 and 2409). The tonicity of the large intestine did not return in any pig in these experiments. In one pig (No. 2409) dead for approximately 4 hours prior to necropsy, the intestine from the midjejunum posteriorly to and including the large intestine was dark red. The color of the intestines in all other pigs was consistently light pink throughout their entire length.

Light Microscopic Lesions

Considerable histologic variation existed in the epithelial cells in the different regions of the small intestine. In the duodenum, the epithelial cells were columnar, and the cytoplasm rarely contained visible vacuoles. The nuclei were centrally located. Many of the jejunal epithelial cells were similar morphologically, but a few contained large apical cytoplasmic vacuoles which appeared to have pushed the nucleus to the base of the cell. The ileum contained more of these vacuolate cells which increased in number as the pigs aged so that, by 48 hours after birth, in many pigs nearly all of the ileal epithelial cells were vacuolate.

The histologic changes observed in the infected pigs were slight and could only be detected when compared to germfree control pigs of the same age. Lesions were first present in pigs which had been infected for 10 hours or longer. The most consistent change observed was edema of the lamina propria (Figure 1) and dilatation of the central lacteal. The latter change was commonly limited to the tips of the villi (Figure 2). These changes were most prominent in the posterior jejunum and ileum. Occasionally, neutrophilic infiltration of the lamina propria of the villi in the jejunum and ileum was observed. This change was more common in the late-infected pigs than in the early-infected pigs. In Pig No. 2409, congestion of

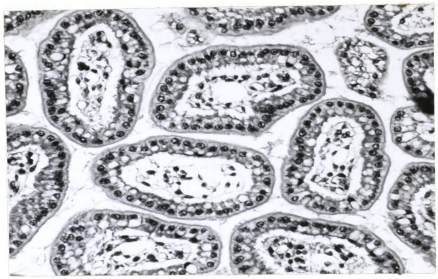

Figure 1. Late-infected Pig 1576. Midjejunum, 17 hours after exposure. Note edema of lamina propria. Hematoxylin and eosin. x350.

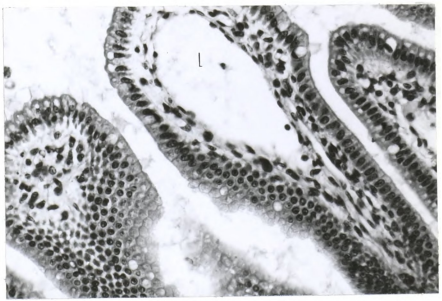

Figure 2. Early-infected Pig 2405. Terminal ileum, 24 hours after exposure. Note dilatation of central lacteal (L) and neutrophils in lamina propria of adjacent villus. Hematoxylin and eosin. x350.

the vessels in the jejunum and ileum was prominent and undoubtedly accounted for the dark red color of the small intestine observed at necropsy (Figure 3).

In the other organs examined, no consistent changes associated with the disease were observed.

Fluorescence Microscopic Findings

All of the methods of tissue preservation gave satisfactory results with the FA technique. The preferred fixative was the mixture of 19 parts absolute ethyl alcohol and one part glacial acetic acid because fixation time was least critical and autofluorescence and nonspecific fluorescence were minimal. Ten per cent buffered formalin caused greater autofluorescence, particularly when fixation time was extended beyond 24 hours, but in less critical studies where photomicrographs were unimportant, it would be a suitable fixative. Thin, flat, serial sections were necessary for this study so paraffin-embedded tissue sections were superior to frozen tissue sections.

The FA-stained *E. coli* organism fluoresced a bright apple-green. A moderate degree of dull yellow to orange autofluorescence of the tissues clearly allowed accurate location of the organism (Figure 4). Adjacent serial sections stained with Giemsa's stain (Figure 5), Gram's stain (Figure 6) or hematoxylin and eosin (Figure 7) demonstrate the difficulty in visualizing the organism

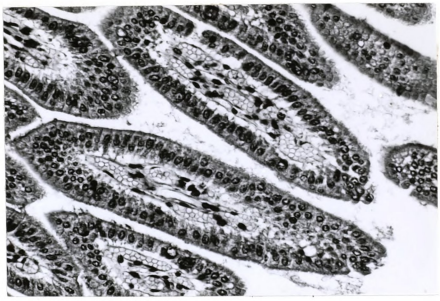

Figure 3. Early-infected Pig 2409. Terminal jejunum, 30 hours after exposure. Pig was dead for 4 hours before necropsy. Note marked congestion of all blood vessels in lamina propria. Hematoxylin and eosin. x350.

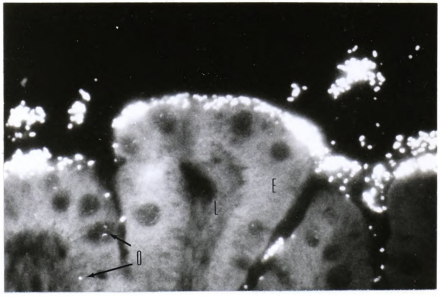

Figure 4. Early-infected Pig 2403. Cecum, 12 hours after exposure. Dull autofluorescence delineates epithelium (E) from lamina propria (L). Note many bright specifically fluorescing *E. coli* organisms on surface and in epithelial cells (O). Fluorescence micrograph. x375.

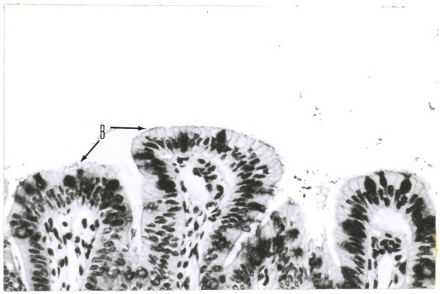

Figure 5. Early-infected Pig 2403. Cecum, 12 hours after exposure. Section adjacent to Figure 4. Bacteria (B) are visible on surface of epithelium but not visible in epithelium or in lamina propria. Giemsa stain. x350.

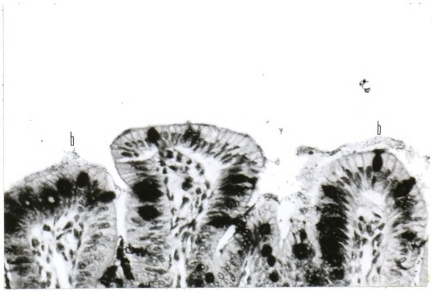

Figure 6. Early-infected Pig 2403. Cecum, 12 hours after exposure, Section adjacent to Figure 5. Bacteria (b) are indistinctly visible on surface of epithelium only. Gram stain. x350.

in the lumen of the intestine and the impossibility of identifying the organism in tissues with conventional stains. Nonspecific fluorescence was occasionally confusing, particularly in the 95% alcohol-fixed tissues at low magnification, but the characteristic morphology of the *E. coli* organism was readily discernible at higher magnification (Figures 8 and 9).

Location of the Organism

Tonsil

A few organisms were present on the mucosal surface of the tonsil within 2 hours after inoculation. Within 4 hours, and at all times following, the surface was covered with a layer of organisms, and the tonsilar crypts were packed with the organisms. Deep within many of the crypts, the typical shape of the organism was not evident. However, fluorescence was brillant, indicating degeneration and lysis of the organism and possibly an accumulation of antigenic by-products of the organism (Figure 10). The organism was never observed within or beneath the epithelium of the pharyngeal mucosa.

Stomach

The organism was observed in the lumen of all the infected pigs. In the late-infected pigs, usually only a few organisms were present in the ingesta and in close

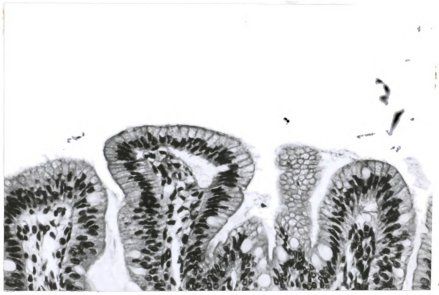

Figure 7. Early-infected Pig 2403. Cecum, 12 hours after exposure. Section adjacent to Figure 6. Bacteria are not visible. Hematoxylin and eosin. x350.

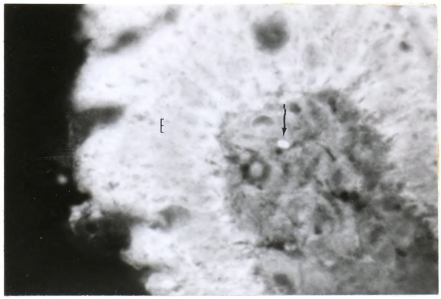

Figure 8. Early-infected Pig 1569. Terminal jejunum, 12 hours after exposure. Epithelium (E). One organism (arrow) is present in the lamina propria. Characteristic morphology of bacterium is clearly demonstrated. Fluorescence micrograph. x850.

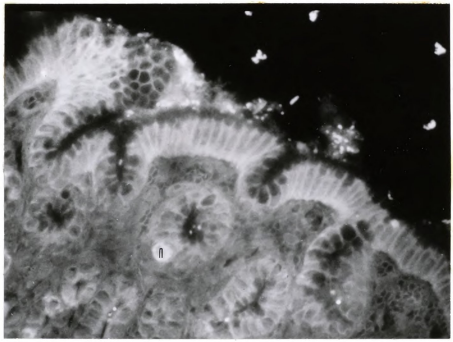

Figure 9. Early-infected Pig 2408. Stomach, 31 hours after exposure. Bacteria are present on mucosal surface as well as in gastric pits. Note nonspecific fluorescence of parietal cells (n). Fluorescence micrograph. x375.

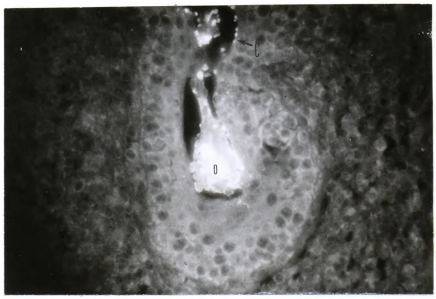

Figure 10. Late-infected Pig 3662. Pharyngeal tonsil, 24 hours after exposure. Note degenerate organisms and antigenic by-products of organisms at base of crypt (O). Intact organisms are present at entrance to crypt (C). Fluorescence micrograph. x375.

association with the glandular mucosa. More organisms were commonly found in contact with the nonglandular mucosa than with the glandular mucosa. In the early-infected pigs, a larger number of organisms was commonly present in the stomach, and often the gastric pits contained bacteria (Figure 9) in contrast to the late-infected animals in which the organism was seldom observed in the gastric pits (Figure 11). The organism was never observed in the gastric epithelium and was only present in the gastric wall of pigs with a bacteremia.

Intestine

In 4 of 7 pigs killed less than 10 hours after exposure, no organisms were observed in the lumen of the duodenum, although organisms were always present in increasing numbers at lower levels of the bowel. The cecum and colon were the most heavily populated regions of the intestine, except in 2 of 3 pigs killed within 4 hours after exposure.

The following observations as to location of the organism in the intestinal tract will be divided into two groups: late-infected pigs and early-infected pigs. Observations on the intestinal wall and other selected organs are summarized in Table 3.

Late-infected pigs.--Numerous organisms were closely associated with the upperhalf of the villi, while only a

Table 3. Fluorescent antibody stained *E. coli* observed in intestinal tissues and other visceral organs.[a]

Hours After Exposure	Duodenum	Midjejunum	Ileum	Colon	Cecum	Mesenteric Lymph Node	Liver	Spleen	Kidney	Lung
Late-Infected (40-120 Hours)										
3-8	0/2[b]	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
10-24	0/7	1/7	3/7	2/7	4/7	2/7	0/7	0/7	0/7	0/7
36-72	0/4	1/4	2/4	0/4	1/4	2/4	0/4	1/4	0/4	0/4
Early-Infected (1-2 Hours)										
3-8	0/5	3/5	2/5	3/5	3/5	1/5	0/5	0/5	0/5	0/5
10-24	1/7	6/7	6/7	6/7	7/7	4/7	3/7	2/7	1/7	1/7
30-50	1/6[c]	3/6	6/6	5/6	5/6	3/6	3/6	3/6	4/6	3/6

[a] 5μ thick sections.

[b] Number positive observations/total number of pigs examined.

[c] One pig dead 4 hours prior to necropsy not included.

few organisms were observed near the base of the villi and in the intestinal glands (Figures 12 and 13). The organisms often appeared to be embedded in the brush border of the intestinal epithelial cells (Figure 14) although this has been shown by electron microscopy not to be a common finding. There was no evidence of phagocytosis of the organism within the intestinal lumen. In the cecum and colon, many organisms were closely associated with the epithelial surface and only a few were present in the crypts of Lieberkühn (Figure 4).

Escherichia coli was never observed in the epithelial cells of the duodenum or in the epithelial cells with large vacuoles in the jejunum and ileum. Occasionally individual organisms were observed in the nonvacuolate cells of the jejunum and ileum. The epithelial cells containing organisms in their cytoplasm were always located in the upper third of the villi. At times, a single *E. coli* was present in the lamina propria and the other mural layers of the jejunum, ileum and large intestine.

In Litter 1, in which ligatures were not placed on either end of the intestinal specimen during necropsy, contamination of the serosa and mesentery was common (Figure 15).

Early-infected pigs.--In this group of animals, the organisms were observed in locations similar to those in the late-infected group. The primary difference was

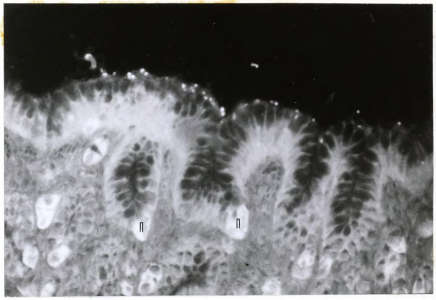

Figure 11. Late-infected Pig 0565. Stomach, 24 hours after exposure. Bacteria are present only on mucosal surface. Note nonspecific fluorescence of parietal cells (n). Fluorescence micrograph. x375

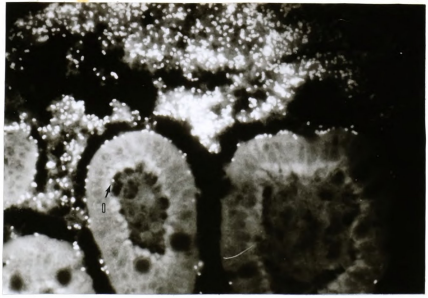

Figure 12. Late-infected Pig 3661. Terminal jejunum, 16 hours after exposure. Numerous organisms are in intestinal lumen and a few are in close association with upper half of villi. Note one organism in subepithelial space (o). Fluorescence micrograph. x375.

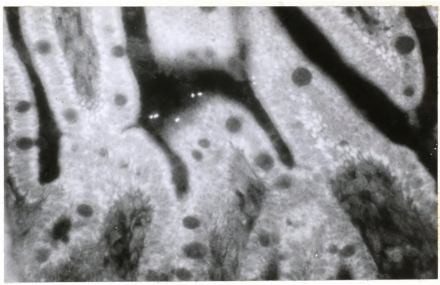

Figure 13. Late-infected Pig 3661. Terminal ileum, 16 hours after exposure. Only a few bacteria are present at the base of villi. Fluorescence micrograph. x375.

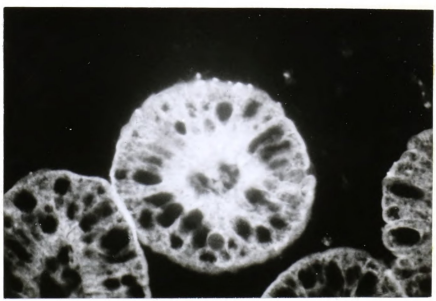

Figure 14. Late-infected Pig 1575. Terminal jejunum, 13 hours after exposure. Tranverse section of villi. Bacteria appear to be partially embedded in brush border. Fluorescence micrograph. x375.

a greater number of organisms in all locations in the early-infected. The only exception was in the lumen of the large intestine where equally large numbers of the organism populated this region of the intestine in the 2 groups. This larger population was particularly evident in all pigs of Litter 3.

Numerous organisms were closely associated with the upper half of the villi as in the late-infected animals. In contrast, however, they were often present in large numbers at the base of the villi and in the intestinal glands of the jejunum and ileum of pigs exposed to the organism for 12 hours or more prior to necropsy (Figure 16). No evidence of phagocytosis of the organism in the intestinal lumen was observed.

A few organisms were present in the duodenal epithelial cells in only 2 pigs (Nos. 2405 and 2407). The organism was frequently found in the villal epithelial cells of the upper third of the villi, in the lamina propria and in other layers of the intestinal wall of the jejunum and ileum of pigs exposed for 8 hours or more (Figure 17). The organism was rarely located in the villal epithelium in the lower two-thirds of the villi or in the epithelium of the intestinal glands. Invasion of the organism was also frequently observed in the cecum and colon in the animals exposed 8 hours or more (Figure 18).

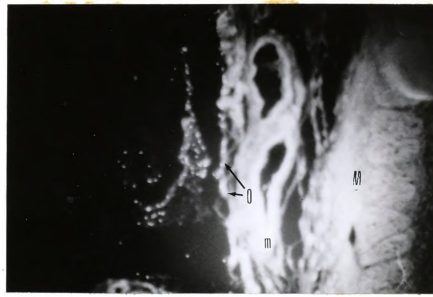

Figure 15. Late-infected Pig 0567. Serosal surface of midjejunum, 72 hours after exposure. Numerous organisms (O) are contaminating mesentery (m). Muscle (M) in intestinal wall. Fluorescence micrograph. x375.

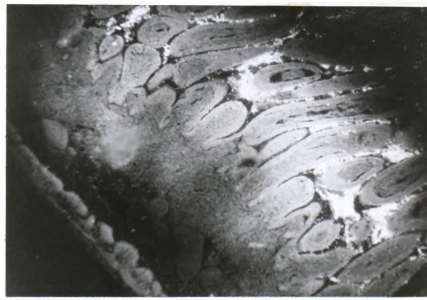

Figure 16. Early-infected Pig 2408. Terminal jejunum, 31 hours after exposure. Spaces between base of villi are packed with organisms. Fluorescence micrograph. x170.

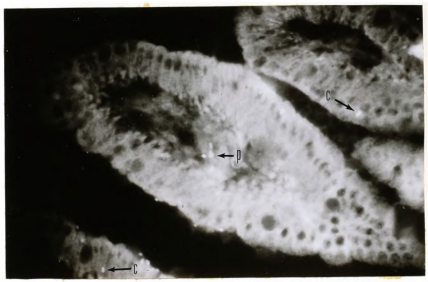

Figure 17. Early-infected Pig 2405. Terminal ileum 24 hours after exposure. Organisms in lamina propria (p) and in villal epithelial cells (c). Fluorescence micrograph. x375.

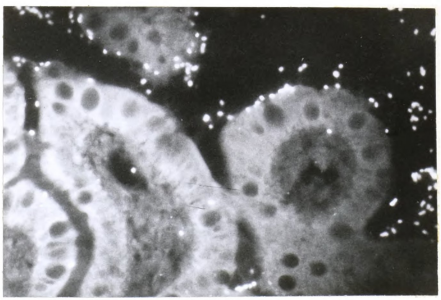

Figure 18. Early-infected Pig 2404. Colon, 16 hours after exposure. Bacteria in close association with epithelial surface and present in epithelial cells and lamina propria. Fluorescence micrograph. x375.

Invasion was most prevalent in the pigs of Litter 3, but even if all 9 pigs recorded in Table 3 were removed (3 from each time period of the early-infected group) a higher percentage of the tissues examined from the early-infected pigs had organisms present than in the late-infected. Also, more organisms were present in the invaded tissues of the early-infected pigs as evidenced both by fluorescence microscopy and by bacterial counts from the other visceral organs.

Other Tissues

In the remaining FA stained tissues (liver, lung, spleen, kidney, mesenteric lymph node and mandibular lymph node) individual organisms were observed (Figure 19). The frequency of these observations correlated closely with the bacterial counts done in Litters 3 and 4. A summary of these observations is recorded in Table 3. The organism was always located in the circulatory system, and no evidence of phagocytosis by the reticuloendothelial system was observed. Only in Pig No. 2409 were clusters of organisms observed in the tissues, indicating that multiplication of the organism had occurred, probably after death (Figure 20).

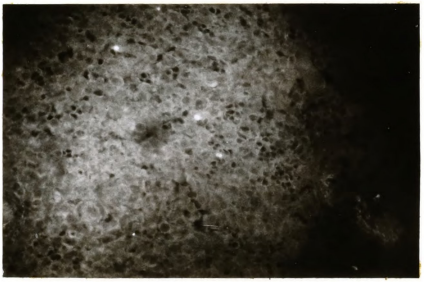

Figure 19. Early-infected Pig 3660. Mesenteric lymph node, 50 hours after exposure. Individual organisms scattered throughout lymphoid tissue. Fluorescence micrograph. x375.

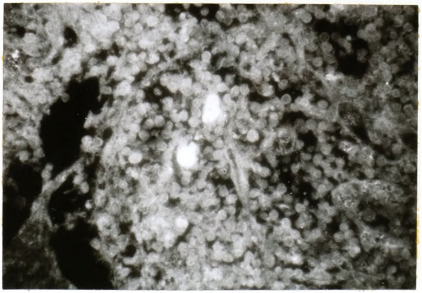

Figure 20. Early-infected Pig 2409. Mesenteric lymph node, 30 hours after exposure. Animal was dead for 4 hours prior to necropsy. Note clusters of organisms in lymphoid tissue. Fluorescence micrograph. x375.

Electron Microscopic Lesions

The pigs used for electron microscopic studies were killed and necropsied between 5 and 52 hours of age and all monocontaminated pigs were exposed to the *E. coli* organism between 1 and 2 hours of age. Ileal epithelial cells from only the apical half of the villi were studied.

Germfree Pigs

The structure of the ileal epithelial cell of the germfree neonatal pig corresponds to that of suckling rats and mice as described by Clark (1959). All the columnar absorptive cells of the ileum had well developed, uniform, closely-packed microvilli projecting from the apical cell membrane (Figure 21). The microvilli were surrounded by a trilaminar membrane which was continuous with the cell membrane. Fourteen straight, electron-dense filaments extended from the tip through each microvillus and continued as well defined rootlets into the apical region of the cell. The filaments formed a consistent ultrastructural pattern in the center of the microvillus. Ten filaments encircled 4 centrally placed filaments. All 14 filaments tended to be grouped in pairs, forming 2 central pairs and 5 peripheral pairs (Figure 22).

A well-defined terminal web was not present, although an indistinct band devoid of cellular organelles existed beneath the brush border. A complex network of tubular

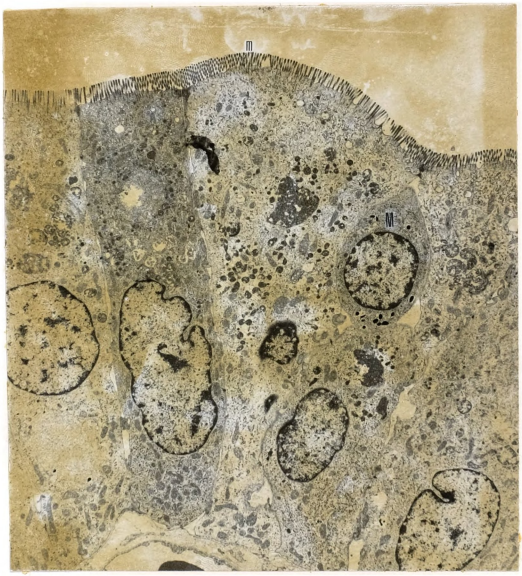

Figure 21. Pig 3665. Ileal villal epithelial cells from noninfected 26-hour-old pig. Cytoplasmic density varies between cells. Uniform microvilli (m) line free surface. Note numerous products of absorption within cytoplasm and migrating lymphocyte (M) between absorptive cells. x7500.

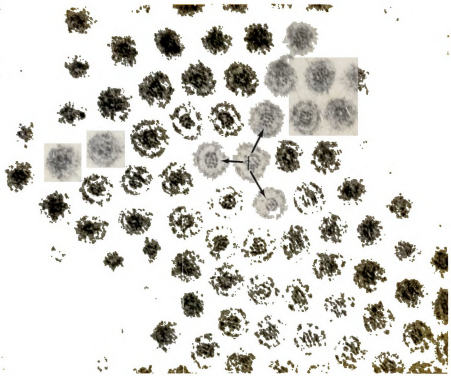

Figure 22. Pig 2411. Transverse section of microvilli parallel to apical cytoplasmic membrane from noninfected 36-hour-old pig. Note arrangement of filaments (f) within microvilli. x32,500.

membranes which occasionally was continuous with the plasma membrane existed in this zone (Figure 23). Portions of these tubules were dilated and may be important in the formation of vacuoles as suggested by Staley *et al.*, (1968).

Mitochondria, smooth and rough endoplasmic reticulum and free ribosomes were evenly distributed throughout the cytoplasm. Some mitochondria were swollen and vacuolated. Membrane-enclosed absorption products were usually limited to the supranuclear region (Figure 21).

Vacuoles in the ileal epithelial cell were variable in both number and size. In the newborn pig less than 10 hours of age, the vacuoles generally were small and numerous, although occasionally only one large vacuole was present. In the older pigs there was a tendency for the vacuoles to be larger, with a single vacuole nearly filling the upper two-thirds of the cytoplasm in the majority of the cells (Figure 24). The large vacuoles appeared to form by coalescence of smaller vacuoles (Figure 23 and 25). As the vacuoles enlarged, the nuclei were pushed to the base of the cells. The mitochondria and endoplasmic reticulum were also displaced so that they were densely packed in the small area immediately surrounding the vacuole (Figure 24). Small vacuoles were bound by a continuous single membrane while the membrane surrounding large vacuoles was often fragmented (Figure 25).

Figure 23. Pig 1573. Ileal villal epithelial cells from noninfected 50-hour-old pig. Apical tubules and vacuoles (A) are numerous. Large vacuoles (V) appear to be forming by coalescence of smaller vacuoles. Adjacent cells are in close apposition and are held together by extensive intercellular interdigitations (I). x10,500.

Figure 24. Pig 2411. Ileal villal epithelial cells from noninfected 36-hour-old pig. Large vacuoles filling upper two-thirds of cytoplasm. Nuclei pushed to base of cells and other cytoplasmic organelles condensed around periphery of vacuoles. Degenerate mitochondrion (m) and membrane fragments present in vacuoles. x9750.

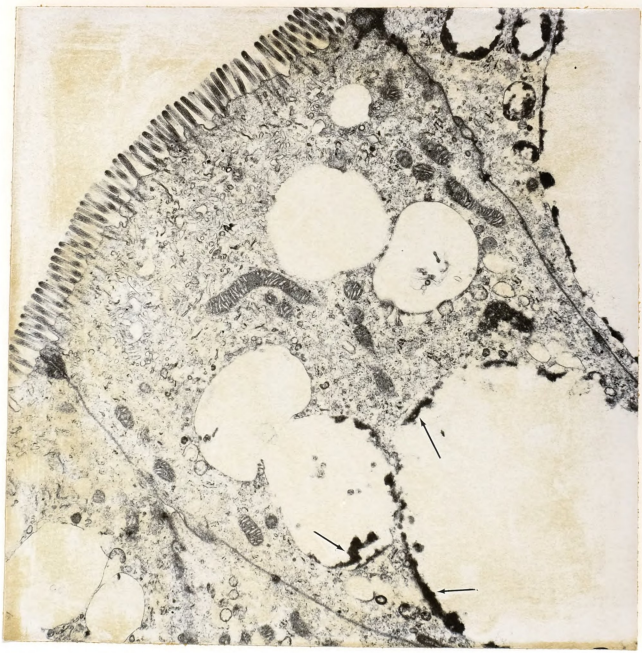

Figure 25. Pig 3665. Apical portion of absorptive cells from noninfected 26-hour-old pig. Smaller membrane-enclosed vacuoles coalescing to form large vacuoles. Electron-dense material has condensed along vacuolar membrane (arrows). x15,000.

The vacuoles were either empty or contained a fine granular material of low electron density. This material was often condensed along the walls of the vacuoles. Degenerating mitochondria and membrane fragments were also present in the large vacuoles (Figure 24).

The cells were usually in close apposition to neighboring cells and held together by intercellular junctions and extensive interdigitations of the lateral cell membranes (Figure 23). Occasionally a small intercellular space existed in which a small amount of granular material of low electron density was present.

Monocontaminated Pigs

The initial changes in the ileal epithelial cells of *E. coli*-infected pigs were observed at 8 hours after exposure. There were few pinocytotic vacuoles and dilated apical tubules, indicating that absorption was lower than in germfree controls of the same age (Figure 26).

Large accumulations of fat were present in many of the ileal epithelial cells. The size of the globules were varied and they filled much of the cytoplasm above the nuclei (Figure 26). Only a few small fat globules were present in the subnuclear cytoplasm.

Some mitochondria were swollen and vacuolate, but no significant or consistent differences were noted in these organelles when compared with control pigs.

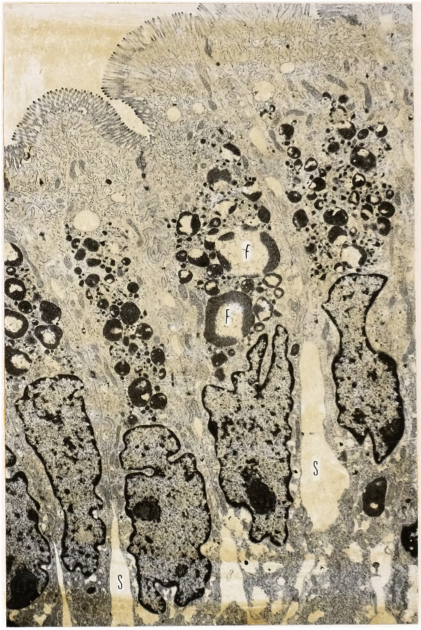

Figure 26. Pig 1568. Ileal absorptive cells from pig infected for 8 hours. Fat globules (F) of various size have accumulated in supranuclear region. Note dilated intercellular spaces (S) at base of cells. x9750.

The lateral intercellular spaces were dilated. This was particularly evident at the base of the epithelial cells. The intercellular interdigitations, junctional complexes and the basal laminae were intact. Little electron dense material was present in the intercellular spaces and in the lamina propria immediately beneath the basal lamina (Figure 26 and 27).

In animals with diarrhea, an increase in degenerating cells was present. The microvilli were short, fragmented, and free within the intestinal lumen. The terminal web was disorganized and vacuolate (Figure 28). The large vacuoles, normally present in many ileal cells, increased in size, leaving only a thin rim of cytoplasm from which the cellular organelles had disappeared. The overall cell size was increased and the cells appeared swollen as evidenced by the decrease or absence of intercellular interdigitations (Figure 29). In a few cells the vacuoles ruptured into the intestinal lumen.

Numerous luminal organisms were present in animals exposed for 8 hours or more. The number of organisms in immediate contact with the brush border was smaller than the fluorescence microscopic studies indicated. It appeared that many organisms were displaced during processing, indicating that no firm attachment existed between the organism and the brush border (Figure 30). Rarely, the organism was lodged between microvilli causing little

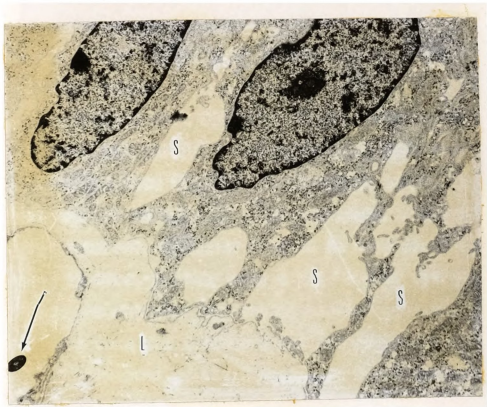

Figure 27. Pig 2405. Base of absorptive cells from pig infected for 24 hours. Dilated intercellular spaces (S) and lamina propria (L) contain little electron dense material. Note organism (arrow) in blood vessel. x10,500.

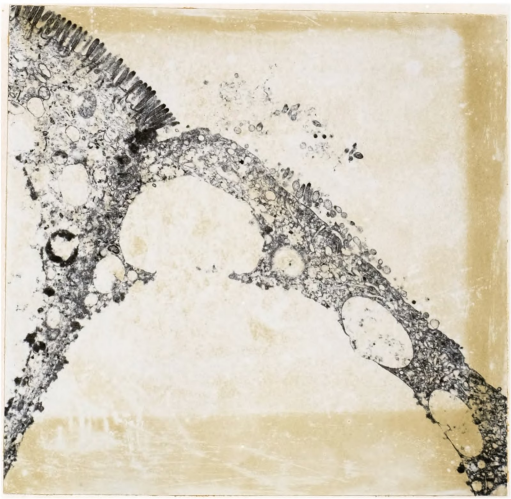

Figure 28. Pig 3660. Apical portion of degenerating ileal absorptive cells from pig infected for 50 hours. Microvilli are short and exfoliating. Terminal web is vacuolate and disorganized. x11,500.

Figure 29. Pig 3656. Apical portion of degenerating vacuolate absorptive cell from pig infected for 15 hours. Vacuole has increased in size (compare with Figure 24) leaving only a thin rim of cytoplasm and loss of intercellular interdigitations. Microvilli are being shed and rupture (arrow) of vacuole appears imminent. x10,500.

or no effect on the cell (Figure 31). Invasion sites were not observed, but infrequently 1 to 4 organisms were present in an epithelial cell. The bacilli were widely separated in the cytoplasm, suggesting that 1 organism entered the cell at a time (Figure 32). They appeared free within the cytoplasm with no evident limiting membrane surrounding the organism (Figure 33). The *E. coli* were never observed in the large vacuolate cells or in the intercellular spaces. Nearly all the invaded cells showed evidence of degeneration. In the severely affected cells, the microvilli were absent or fragmented; the mitochondria were few in number, often swollen and lacking in contrast; and the cytoplasm was opaque and foamy (Figure 33).

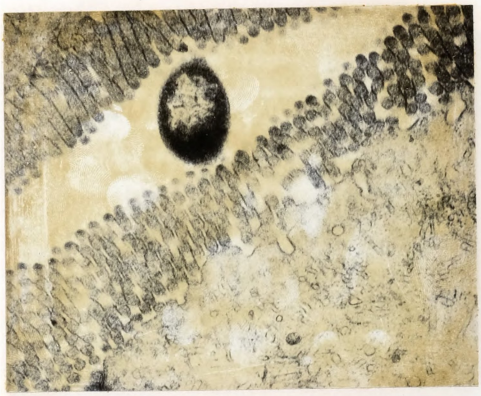

Figure 30. Pig 1570. Microvillal border of ileal absorptive cell from pig infected for 24 hours. Organism in close association but not attached to microvilli. Organism causing no visible damage to cell. x20,500.

Figure 31. Pig 2404. Apical ends of ileal cells from pig infected for 16 hours. Organisms are in direct contact with brush border. Note one organism (arrow) wedging between microvilli but causing no damage. x11,500.

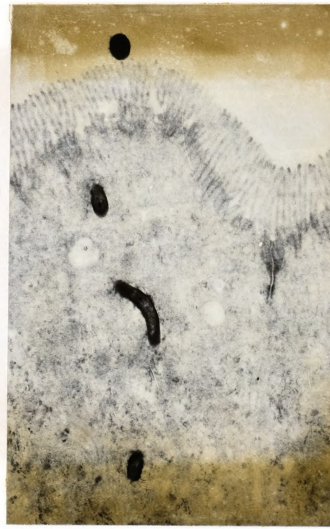

Figure 32. Pig 2405. *E. coli* organisms within degenerate epithelial cell from pig infected for 24 hours. Organisms are widely spaced in cell and free within cytoplasm. x12,500.

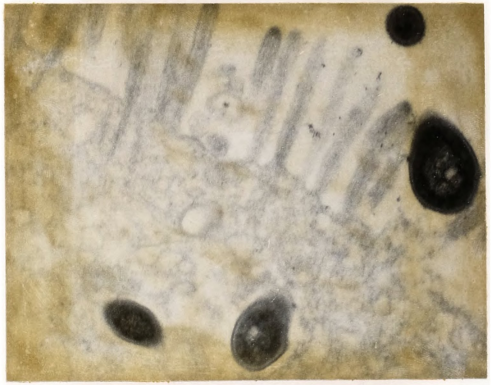

Figure 33. Pig 2406. *E. coli* organisms within degenerate epithelial cell from pig infected for 31 hours. Cytoplasm is foamy and vacuolate. Microvilli are fragmenting and sloughing. There is no evidence of a limiting membrane around the organisms. x26,000.

DISCUSSION

Clinical, Gross and Microscopic Findings

The pathogenicity of *E. coli* 0138:K81:NM in colostrum-deprived gnotobiotic pigs was confirmed in this series of experiments. All pigs orally exposed to approximately 3 million organisms, if allowed to live the necessary 12- to 16-hour incubation period, developed the typical signs of neonatal enteric colibacillosis. These results are in agreement with those of Christie (1967, 1969) for this serotype and with other enteropathogenic serotypes studied by Kohler and Bohl (1966) and Kohler (1967) in gnotobiotic pigs.

According to Dunne (1964) pigs infected with pathogenic strains of *E. coli* usually have anorexia. Loss of appetite does not appear to be a significant clinical sign in gnotobiotic pigs infected with this serotype of *E. coli*. Only in severely affected pigs with a bacteremia and whose overall condition was rapidly deteriorating was anorexia observed. In pigs less severely affected, with none or only an occasional organism present in the visceral organs, anorexia was not present. Therefore, anorexia may only be an indication of systemic bacterial invasion or

general deteriorating clinical condition rather than a specific clinical sign associated with enteric colibacillosis.

The gross lesions were similar to those previously described by a number of authors. It is interesting to note the absence of congestion or inflammation in the posterior regions of the small intestine and the entire large intestine in pigs necropsied immediately after death. On the other hand, in one pig necropsied 4 hours after death, the posterior jejunum, ileum, and large intestine were dark red and congested, suggesting enteritis. Similar observations have been reported by other intestigators, indicating that rapid changes occur in the intestinal tract immediately before or soon after death caused by enteric diseases (Smith and Jones, 1963; Kohler and Bohl, 1966). These findings emphasize the importance of recording the time elapsed between death and necropsy, when associating gross lesions of the intestinal tract with enteric diseases.

In enteric colibacillosis of gnotobiotic pigs, neutrophilic infiltration of the villi is the most common histopathologic lesion reported (Kohler, 1967; Christie, 1967; Staley, *et al.*, 1969). Neutrophilic infiltration of the villi in these experiments was more common in the late-infected than in the early-infected pigs, indicating that pigs by 3 to 4 days of age have developed a more

responsive cellular defense mechanism to bacteria or bacterial toxins than the 1- to 2-day-old pigs. The number of neutrophils in the villi was small and was, in fact, less than that which is present in the villi of normal, healthy conventionally raised pigs. Kenworthy and Allen (1966) also reported an increased cellular infiltration in the lamina propria of the villi of pigs raised in a normal environment as compared with germfree, monocontaminated or duocontaminated gnotobiotic pigs. Neutrophilic infiltrations may indicate a nonspecific host response to the presence of bacteria as suggested by Kohler (1967). There was no correlation between the presence of *E. coli* in the epithelial cells or the lamina propria of the villi with the presence of neutrophils in the core of the villi.

Fluorescence Microscopy

Previous usage of fluorescence microscopy in colibacillosis has been limited to the identification of pathogenic *E. coli* in heat-fixed fecal smears. To the knowledge of the author, this is the first report of utilizing the FA technique to localize and identify *E. coli* in frozen and fixed tissues. Other investigators (Taylor, *et al.*, 1958; Kohler, 1967; Losos, 1964; Staley, *et al.*, 1969) have described *E. coli* in Gram or Giemsa stained tissues, but the efficacy of these stains appear

limited as shown in Figures 5 and 6. Many organisms in the Gram or Giemsa stained tissues appeared identical to portions of cells or to cellular organelles and therefore impossible to identify accurately.

The choice of tissue preservative does not appear to be critical. The *E. coli* organism retained its antigenicity and fluorescence was of equal intensity in chemically fixed tissues, frozen tissues and in heat-fixed smears. This is due to the stability of the polysaccharide antigen of *E. coli*. According to White (1961), organisms possessing polysaccharide antigens can be stained with fluorescent labeled antibodies in sections of unfixed frozen tissue, fixed frozen tissue and fixed tissue embedded in paraffin. However, Schmidt (1952) reported that certain polysaccharide antigens are water soluble and procedures which use an aqueous phase could not be used for the FA technique. Similarly, Winter and Moody (1959) had no success when 10% formalin was used for fixation of *Pasteurella pestis*; but 5% formalin was a suitable fixative. Therefore, as recommended by White (1961), each antigen should be tested to determine the best fixative.

The degree of autofluorescence was similar between the frozen tissues and the acetic acid-alcohol-fixed tissues. Autofluorescence was most evident in the formalin-fixed tissues, but it did not seriously interfere with the visualization of the bright, specific fluorescence of the

bacteria. The addition of a counterstain, rhodamine, as recommended by Smith *et al.* (1959), improved the contrast in color between tissue autofluorescence and the specific fluorescence of the bacteria.

The parietal cells of the stomach and leukocytes occasionally fluoresced brightly and interpretation was not clear at low magnification, but the characteristic morphology of either the tissue cell or of the bacterium could be distinguished at higher magnifications. This nonspecific fluorescence was least evident with tissues fixed in alcohol-acetic acid and most evident in tissues fixed in 95% ethyl alcohol. It was at no time sufficiently disturbing to warrant the absorption of the labeled globulins by tissue powders as recommended by Coons and Kaplan (1950) to decrease nonspecific staining by the labeled globulins.

No specific fluorescence was observed in tissues from the germfree control pigs which were stained with labeled specific globulin. Also, no specific fluorescence was present in infected tissues stained with normal (non-immune) labeled globulin. These procedures were used as tests for the specificity of the staining reaction, and their results indicate that the fluorescein-labeled specific globulin reacted only with the *E. coli* organism.

The specificity, sensitivity and rapidity of the FA technique as applied to *E. coli* has been demonstrated

by other investigators (Whitaker *et al.*, 1958; Thomason *et al.*, 1959). Because these experiments have shown that this technique can be now applied to frozen and fixed tissues, particularly formalin-fixed tissues, it may warrant use in diagnostic laboratories to rapidly identify pathogenic strains of *E. coli* in fixed tissues.

It was clear from these studies that *E. coli* 0138:K81:NM was capable of inhabiting the lumen of the entire alimentary tract of the neonatal gnotobiotic pig. High numbers of bacteria populated the oral cavity. Decreased numbers of microorganisms were present in the stomach and duodenum, with increasing numbers observed as more distal levels of the intestine were examined.

The mucosa of the pharyngeal tonsil was layered with the organism, and many organisms packed the tonsilar crypts. The organism appeared to become trapped in the depressions, and many degenerated, as evidenced by the presence of lysed organisms in the depths of the crypts. In contrast to the findings of Fey *et al.* (1962) in colostrum-deprived calves, in which they concluded that *E. coli* invaded the pharanygeal mucosa, at no time was the organism found in the epithelial cells or in the subepithelial tissues. Therefore, it appears that invasion through the pharyngeal mucosa in gnotobiotic pigs by this serotype does not occur.

Lysis of the bacteria was rarely observed in regions of the digestive tract other than in the crypts of the tonsil. This was probably due to the muscular contractions in these organs which cause movement of ingesta and intraluminal organisms through the tract. This movement could have also caused fragmentation of the degenerate bacteria into particles too small to be detected microscopically and, therefore, only intact bacteria were observed.

Diffuse background specific staining, which one might expect to see due to *E. coli* endotoxin or enterotoxin, was not present in any of the tissues. Failure to observe such staining can be explained in 1 of 3 ways: first, the toxin produced by the organism is antigenic but present in such low concentrations that it cannot be detected by fluorescence microscopy; second, the toxin is not antigenic and therefore not stained by the specific labeled globulin; third, the toxic material is lost in processing. It appears from previous reports of other investigators that the toxic material produced by the organism is antigenic (Kohler and Bohl, 1966; Kohler, 1967). They observed no diarrhea in pigs which had received immune serum orally, nor was there a decrease in the number of organisms cultured from the intestinal tract. This suggests that the toxin liberated by the organism was neutralized by the serum. If the toxin was neutralized

by the specific serum, it must therefore be antigenic. A similar conclusion can be reached from the work of Gyles and Barnum (1967). Specific OK immune serum prevented dilatation of ligated intestinal loops infected with live *E. coli*, but the number of *E. coli* in the loops was not affected. The antibodies must have neutralized the toxin. It appears unlikely that the toxin was lost in processing because, deep within the tonsilar crypts where the organism was trapped along with its by-products, diffuse staining of lysed organisms and/or their toxins was commonly observed.

Therefore, present evidence suggests that the toxin must be antigenic but present in such low concentrations within the tissues of infected animals that it cannot be detected by fluorescence microscopy. Miler *et al.* (1964) also concluded that the level of *E. coli* specific antigen (endotoxin) in the organs of newborn piglets infected with *E. coli* was too low to be detected by the hemagglutination-inhibition test.

The numbers of organisms in the lumen and in the gastric pits of the stomach were higher in the early-infected than in the late-infected pigs. The results of Smith and Jones (1963) can explain these findings. They reported the pH of the stomach of the 1- to 2-day-old pig to be insufficiently low to prevent proliferation of the *E. coli* organism in this organ. It even appears from this

study that the acidity within the gastric pits was not sufficiently high to prevent proliferation and colonization in these depressions of the mucosa of the 1- to 2-day-old pig. In the pigs 3 to 4 days of age or older, either the acidity in the gastric pits has increased and prevents proliferation of the organism, or the flow of secretions from the gastric glands has increased and washes the organism from these depressions of the mucosa. The increase of organisms associated with nonglandular mucosa was also probably due to decreased acidity in this region or to the absence of secretions which would tend to wash the organisms from the mucosal surface.

In the small intestine, the organism was closely associated with the upper half of the villi as also reported by Staley *et al.* (1969). As the number of luminal organisms increased in the posterior regions of the small intestine, there was a corresponding increase in the number of organisms associated with the villi. During fluorescence microscopic studies, the bacteria appeared to be embedded in the brush border of the epithelial cell. However, electron microscopic studies revealed the organism to be closely associated with, but usually not embedded in, the brush border. This discrepancy was probably due to the labeled antibodies attaching to the periphery of the organism and making the organism appear larger than it actually is. Therefore, part of the fluorescing

antibodies are superimposed over the brush border, making the bacteria appear to be embedded in the brush border.

It is clear from these studies that it is unnecessary for the intact organism to invade the villal epithelium to produce diarrhea. Diarrhea was often observed in animals in which few or no organisms were present within the tissue cells. Therefore, it appears likely the organism produces a toxic agent which causes the diarrhea. A toxin produced by *E. coli* has been shown to cause fluid accumulation, similar to that seen with living organisms, when placed in ligated intestinal loops (Taylor and Bettelheim, 1966; Smith and Halls, 1967b). Also, filtrates of enteropathogenic strains of *E. coli* administered intragastrically to young pigs produced diarrhea in 1-1/2 to 3 hours. The diarrhea lasted for 3 to 10 hours (Kohler, 1968).

Kohler (1967) reported bacteria frequently present in the villi, submucosa and mesentery of gnotobiotic pigs. In this study the organism was often located in the villi and submucosa but rarely present in the mesentery of pigs in which particular care was taken to prevent contamination of the serosa and mesentery. Once the organisms reach the submucosa, they appear to be rapidly removed by the blood vascular system.

In the early-infected pigs, which showed pronounced atony of the small intestine grossly, the organism was also

observed in large numbers at the base of the villi and in the intestinal glands. This period of suspected hypomotility, in effect, destroys the normal cleansing mechanism of the intestine, allowing abnormal numbers of organisms to accumulate at the base of the villi and in the intestinal glands.

Electron Microscopy

The ultrastructural changes in the ileal epithelial cells of infected animals not invaded by bacteria were slight when compared with the germfree control pigs. The most significant changes in the early stages of infection were the large accumulations of fat in the supranuclear region and the dilatation of the intercellular spaces. Similar accumulations of fat were reported in lethal intestinal viral infection in mice (Biggers, 1964), in celiac disease in man (Rubin *et al.*, 1966) and in transmissible gastroenteritis of swine (Thake, 1968). Fats are absorbed as monoglycerides and fatty acids by simple diffusion through the apical plasma membrane. The monoglycerides and fatty acids are reformed into triglycerides inside the epithelial cell (Porter, 1969). The triglycerides then pass through the intracellular channels, leave the cell at the lateral cell walls, pass through the lamina propria and are picked up by the lymphatic vessels (Palay and Karlin, 1959). Fat droplets were not observed

in the lateral intercellular spaces so it appears that the products of triglyceride hydrolysis were formed in the intestinal lumen, diffused into the cell, and were reformed into triglycerides; but the mechanism for discharge from the cell was not functioning. Hence, fat accumulated in the cytoplasm of the epithelium.

The presence of pinocytotic vesicles and foamy material of low electron density in cytoplasmic vacuoles in normal cells and often in dilated intercellular spaces are indicative of protein absorption in suckling pigs (Sibalin and Björkman, 1966). In the infected pigs there was a decrease in pinocytotic vesicles and no material was observed in the dilated intercellular spaces, suggesting absorption of proteins was decreased.

Kenworthy *et al.* (1967) reported that all the jejunal epithelial cells in *E. coli*-infected weanling pigs showed degenerative changes identical to those observed in clinically healthy animals. Similar regressive changes were observed in infected pigs in this study, but by no means were all the ileal epithelial cells affected. In fact, the majority of cells appeared normal, and the presence of increased numbers of degenerating cells was recognized only when compared to germfree controls. Sloughing of the microvilli was observed infrequently and did not appear related to the presence or absence of bacteria in the immediate area. Staley *et al.* (1969) suggested

that *E. coli* must be fimbriated to cause degeneration and exfoliation of the microvilli. *E. coli* (0138:K81:NM) is a nonfimbriated organism and did not develop fimbriae during the course of these experiments.

Because of the small number of microorganisms in the epithelial cells, invasion sites were not observed. Unless the organism is fixed as it is entering the cell, evidence of invasion may not be present because the brush border and plasma membrane are capable of rapid regeneration (Takeuchi, 1967). The organisms appeared to penetrate the cells individually because the majority of the invaded cells contained only one organism. When more than one microorganism was present, they were never in close proximity to each other. The intraepithelial microorganisms were always free within the cytoplasm, in contrast to the membrane-enclosed *E. coli* reported by Staley *et al.* (1969).

The organisms were present only in epithelial cells showing regressive changes. The degenerating cells appeared identical to cells undergoing the normal necrobiotic process. It could not be determined if the intraepithelial organisms caused these degenerative changes or if the organism tended to invade only degenerating cells. Taylor *et al.* (1958) stated that dead or degenerative cells were infiltrated with *E. coli*, and extensive invasion of living cells was never observed.

Relationship of Intestinal Motility to Lesions

The development of colibacillary diarrhea is undoubtedly a very complex mechanism. The relationship of intestinal peristalsis or blood flow to the development of the disease has not been studied. In the ligated intestinal loop inoculated with enteropathogenic *E. coli*, movement of bacteria through the small intestine by peristalsis is prevented and lesions similar to those of colibacillary diarrhea are readily and consistently reproduced. In experimental reproduction of enteric shigellosis and salmonellosis, it is necessary to inhibit intestinal motility with opiates to reproduce these diseases (Formal *et al.*, 1963; Kent *et al.*, 1966).

The following discussion considers some of the possible relationships of intestinal motility and blood flow to the development of enteric colibacillosis due to *E. coli* 0138:K81:NM.

A consistent finding which appeared related in both the light and electron microscopic studies was the increase in intercellular and subepithelial spaces and dilatation of the central lacteal. It is interesting to speculate on the mechanism causing these lesions.

One function of the junctional complexes (*zona occludens*, *zona adherens* and *macula adherens*) is to act as a diffusion barrier, thereby preventing free movement

of substances from the intestinal lumen into the intercellular spaces and also the reverse flow of substances from the intercellular spaces into the intestinal lumen (Farquhar and Palade, 1963). In this study, the junctional complexes were intact; therefore, free movement of fluid from the intercellular spaces to the intestinal lumen was not possible. The normal rate of absorption was decreased because there was a decrease of pinocytotic vesicles. Thus, the intercellular spaces probably were not the result of the normal movement of absorption products through the lateral cell membranes and accumulating in the intercellular spaces before passing into the vascular system. The most likely origin of this fluid, then, was from the vascular system. These microscopic changes were present at approximately the same time after exposure as atony and suspected hypomotility of the small intestine. Muscular contraction and relaxation normally assist the movement of fluid through the vascular system. A decrease or absence of muscular activity as seen in these studies could cause an accumulation of intra- and/or extra-vascular fluid.

In addition, Zweifach (1964) has reported that high levels of *E. coli* endotoxin administered intravenously to rabbits and rats caused constriction of the mesenteric venules and slowing of blood flow through the capillaries. Damage to the capillary endothelium and alteration of the

permeability of the small vessels were also associated with high levels of endotoxin. These changes in blood flow and alteration of permeability could cause an imbalance between the hydrostatic and osmotic pressures in the intestinal villi where the highest concentrations of endotoxin are most probably located in enteric colibacillosis. The net effect of this imbalance would be an accumulation of fluid in the lamina propria, extending into the intercellular spaces. In light and electron microscopy this excess fluid would appear as dilated subepithelial and intercellular spaces.

The neonatal animal is more susceptible to enteric colibacillosis than older animals. By oral inoculation with *E. coli*, Smith and Halls (1967a) were able to produce diarrhea in calves less than 20 hours old but failed to produce clinical signs of the disease in 3-day-old calves. They concluded that to cause diarrhea the organism must proliferate in the anterior small intestine and suggested that the physiological state of the epithelium of the small intestine in the very young calf may permit adhesion of the enteropathogenic *E. coli*. It appears from both the fluorescence and electron microscopic studies that *E. coli* 0138:K81:NM does not adhere to the epithelium. This was particularly evident in FA stained sections of the anterior small intestine where very few and occasionally no organisms were observed in the lumen or in immediate contact with the

villal epithelial cells. It was generally true throughout the entire intestinal tract that relatively few organisms were in contact with the mucosa when compared with the number of organisms free within the intestinal lumen. Therefore, this indicated that adhesion of the bacteria to the epithelium was not an important factor with this strain of *E. coli*.

It appears that the neonatal animal is also more susceptible to the effects of *E. coli* enterotoxin than older animals. Kohler (1968) was more successful in producing diarrhea by filtrates of *E. coli* in pigs 6 to 24 hours old than in 2- to 5-day-old pigs. Enterotoxins appear to be large molecular substances because they are nondialyzable (Smith and Halls, 1967b) or only partially dialyzable (Kohler, 1968). The newborn pig is capable of absorbing intact protein for 24 to 36 hours after birth (Payne and Marsh, 1962). The level of enterotoxin absorbed may be related to this function of the intestine. In the very young pig, a higher level of enterotoxin may be absorbed, resulting in more severe and persisting effects on the intestine. In this series of experiments most of the early-infected pigs were more severely affected than the late-infected pigs.

The enterotoxin appeared first to decrease the motility of the intestine, and if the hypomotility persisted, the number of organisms increased at the base of

the villi. Invasion of the epithelial cells in the upper third of the villus was then commonly seen, resulting in bacteremia. The late-infected pigs were less severely affected than the early-infected, possibly due to absorption of lower levels of enterotoxin. In the late-infected pigs atony of the small intestine subsided, the number of organisms did not increase to a significant level at the base of the villi, and invasion of the epithelium was less common.

A series of events may be proposed from these findings. As a high population of organisms colonize at the base of the villi because of hypomotility, a corresponding high level of enterotoxin is released. Damage to the villal epithelial cells at the lower levels of intestinal villus occurs. These damaged epithelial cells are possibly more susceptible to invasion by *E. coli* 0138:K81:NM, as indicated by the electron microscopic studies and supported by the conclusions of Taylor *et al.* (1958). Therefore, before the degenerate cells are extruded at the tips of the villi, bacteria pass through them, gaining access to the lamina propria with bacteremia and death the eventual outcome.

Invasion was most common in the intestine from the midjejunum posteriorly. This was most likely associated with the increased cellular damage of the epithelium due to increased enterotoxin released by the large population

of bacteria in these regions. Also, increased numbers of bacteria present in the lumen and randomly contacting the mucosa increase the chances of invasion in these regions over areas such as the duodenum, where only small numbers of bacteria are usually present.

It may be that further studies relating intestinal motility and blood flow through the intestine would significantly contribute to our understanding of the pathogenesis of colibacillary diarrhea.

SUMMARY

Four experiments using 40 gnotobiotic pigs were conducted to study the light and electron microscopic lesions produced by *E. coli* 0138:K81:NM. The pigs were exposed orally to approximately 3 million enteropathogenic *E. coli* organisms at 1 to 2 hours of age (early) or at 40 to 120 hours of age (late).

The organism was identified and localized in the tissues by the fluorescent antibody (FA) technique. Hyperimmune anti-*E. coli* serum, produced in rabbits, was labeled with fluoresceinisothiocyanate and used to stain the tissue sections.

It was found that the FA technique could be applied to paraffin-embedded tissues fixed in 95% alcohol, 10% buffered formalin or a mixture of absolute ethyl alcohol and acetic acid as well as to frozen tissues. The *E. coli* organism fluoresced a bright apple-green against a dull orange-red autofluorescence of the tissues. No diffuse specific staining due to antigenic *E. coli* endotoxin or enterotoxin was observed.

The organism colonized the entire digestive tract. It was present in larger numbers in the stomach and duodenum of the early-infected pigs than in the late-infected

pigs. The organism was commonly associated with the upper half of the villi and rarely populated the intestinal glands or the intervillus spaces at the base of the villi in the late-infected pigs. In the early-infected pigs which clinically were more severely affected, the organism was present in larger numbers at the base of the villi and in the intestinal glands.

It was concluded that the organism does not have to invade the intestinal epithelium to cause diarrhea. The organism did not invade the pharyngeal or gastric mucosa, rarely invaded the epithelium of the duodenum and invaded the epithelium in increasing frequency at more distal levels of the intestine. Invasion of the epithelium occurred only in the upper third of the villi and was much more frequent in the early-infected pigs than the late-infected pigs.

Light microscopic lesions of the intestine were mild. They were first noted at 10 hours after exposure and primarily consisted of edema of the lamina propria and dilatation of the central lacteal.

The initial ultrastructural changes in the ileal epithelium, present at 8 hours after exposure, were a decrease in pinocytotic vesicles, dilatation of the intercellular spaces, and accumulation of fat globules in the cytoplasm. As the disease progressed, there was an increase in number of cells showing regressive changes

identical to the degenerative changes present in cells of the germfree controls undergoing necrobiosis.

The presence of organisms in immediate contact with the brush border did not visibly damage the microvilli. There was no evidence of attachment between the organisms and the microvilli. The organisms appeared to enter the cells one at a time and were always free within the cytoplasm. Bacteria were found only in cells with evidence of degenerative changes.

In the severely affected pigs, the posterior jejunum, ileum and large intestine were flaccid and remained in this condition until the pigs were killed, but in the less severely affected pigs the atony of the small intestine was temporary. In the pigs in which atony persisted, the organism colonized at the base of the villi and in the intestinal glands. There was also an increase in epithelial intercellular spaces along with edema of the lamina propria. It is proposed that these changes were associated with hypomotility of the small intestine.

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VITA

The author was born November 23, 1933 in Dyersville, Iowa. His primary and secondary education was completed in that locale in 1951.

The author entered Iowa State University in September, 1951. While enrolled at this institution he met Janet Elizabeth Hoben whom he married at Greene, Iowa in December, 1956. In June, 1957, he received the D.V.M. degree. The author practiced 6 months as an associate of Dr. J. N. Hunt in Mt. Pleasant, Iowa. In December, 1957, he entered into partnership in a large animal practice with Dr. James P. Hoben at Greene, Iowa.

In August, 1964, the author accepted a position as an instructor in the Department of Anatomy at Michigan State University. He matriculated as a part-time graduate student and received his M.S. degree in June, 1966. He then enrolled in the Department of Pathology and was granted an NIH post-doctoral fellowship in July, 1968.

The author is a member of the A.V.M.A., Phi Kappa Phi, Phi Zeta, and Sigma Xi.