OBSERVATIONS ON EXPERIMENTAL COLIBACILLOSIS IN THE YOUNG GNOTOBIOTIC PIG

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CHAPTER I

INTRODUCTION

Diarrheal diseases are considered to be a major problem of infants and children (Anon., 1966a). In some geographic regions diarrhea in early childhood is a syndrome which is often encountered in pediatric practice (Anon., 1966b). Most authors report a high mortality rate, which may reach 80% in some outbreaks (Wilson and Miles, 1964). Escherichia coli is considered to be a primary etiologic agent in diarrhea of newborn humans, calves, and swine (Edwards and Ewing, 1962; Gay, 1966; Anon. 1959, 1960). Surveys in England indicate that E. coli is the organism most frequently associated with diseases of pigs less than 3 weeks old (Anon., 1960). Several distinct disease entities in swine have been attributed to E. coli, viz., neonatal septicemia, neonatal colibacillary diarrhea, weanling colibacillary diarrhea, and edema disease (Nielsen, Moon, and Roe, 1968; Barnum, Glantz and Moon, 1967). This study concerns only the first 3 syndromes.

Disorders characterized by diarrhea commonly occur prior to maturation of an effective intestinal barrier. Factors which influence the development of this barrier include: nutrition, exposure to colostrum early in life,

development of the reticuloendothelial system, differentiation of the intestinal epithelial cells, colonization of the intestine by bacteria, and development of local immunity, if this does indeed occur.

The germfree pig has proved to be a valuable tool in the study of diarrheal disease. Some advantages of the gnotobiotic pig as an experimental animal are: advanced development at birth (i.e., it can walk almost immediately and can feed itself within hours after delivery); litter size which provides within-litter control animals; and the close anatomic and physiologic similarities to human gastrointestinal, vascular, and other organ systems (Landy, et al., 1961). The gnotobiotic pig is particularly suitable for studies of infectious disease and the development of resistance. They are free of demonstrable antibody at birth, do not harbor microorganisms which might alter the course of the disease, and maintenance of controls is facilitated by complete isolation (Kohler and Bohl, 1966a).

The purpose of this investigation is to contribute to knowledge of development of resistance to neonatal disease, utilizing gnotobiotic piglets infected with Escherichia coli as a model system.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Intestinal Resistance of the Young Pig

Little is known about the development of resistance to intestinal infections in the young pig. Under proper conditions, colibacillary diarrhea can be experimentally induced in the neonatal pig (Kohler, and Bohl, 1966a; Kohler, 1967a). However, in older pigs it has not been possible to reproduce the disease consistently (Lecce and Reep, 1962). The complex nature of gastrointestinal maturation has resulted in failure to elucidate the factors responsible for the development of resistance in the young animal.

Non-Specific Factors Associated with Resistance

Many factors are responsible for controlling the proliferation of bacteria in certain tissues. Irregularity in the function of these factors may predispose an animal to disease. Taylor (1966) reported that Escherichia coli could be isolated from the duodenum of infants with colibacillary diarrhea, an area which is usually sterile in the healthy infant. Colonization and proliferation of enteropathogenic E. coli in the proximal small intestine is an essential feature of colibacillary diarrhea in pigs (Kenworthy and

Crabb, 1963; Smith and Jones, 1963). Smith and Jones (1963) report that a relatively high pH in the proximal intestine of the neonatal pig is conducive to proliferation of E. coli in the anterior small intestine. Kohler (1967a) reported the gastric pH of infected 4 to 6-day-old gnotobiotic pigs was 2.5 to 4.5, and clinical colibacillosis was observed in these E. coli-infected pigs.

Nielsen, Moon, and Roe (1968) indicate that glandular secretions supply large amounts of sterile fluid to the proximal small intestine. They postulated that normal reduction in numbers of luminal bacteria in the anterior small intestine may be due to "washing out" by glandular secretions. Intestinal motility has been emphasized as a factor in controlling bacterial populations in the anterior small intestine. Formal, et al., (1964) report that intestinal motility is responsible for flushing of bacteria toward the ileum, and this phenomenon is effective in preventing Shigella invasion in adult guinea pigs. Studies carried out utilizing the ligated-loop model, a technique in which ligatures are placed at each end of a short segment of intestine and the resulting loop injected with bacteria, reveal that extreme stasis magnifies the lesion of colibacillary diarrhea (Nielsen, Moon, and Roe, 1968).

The intestinal permeability to large proteins is well established in certain neonatal aminals (Payne and Marsh, 1962a; Miller, et al., 1962; Pierce and Smith, 1967; Staley, Jones and Corley, 1969b). Intestinal permeability

is the means by which passive immunity is conveyed to the neonatal ungulate via colostrum absorption during the first few hours of life (Brambell, 1958; Miller, et al., 1962). The loss of the ability to absorb antibodies, termed closure, occurs early in the life of the neonate (Brambell, 1958; Payne and Marsh, 1962a). Permeability of swine intestinal epithelium to colostral antibodies declines exponentially during the first 36 hours after birth (Speer, et al., 1959). According to Payne and Marsh (1962a), however, closure had not occurred at 106 hours after birth in pigs which had been starved. Absorption of immunoglobulins is thought to be a nonselective process since heterologous proteins are absorbed by the baby pig (Ibid.). Previous investigations have revealed that absorption of intact proteins may be related to immaturity of the intestinal epithelial cell and associated enzyme systems (Staley, 1969). Ultrastructural changes in duodenal and jejunal absorptive cells occur simultaneously with the development of a barrier to intact protein uptake (Staley, Jones and Corley, 1969b); and Mattison and Karlsson, 1966). Loss of intestinal permeability has been associated with the appearance of alkaline phosphatase in the microvilli of the epithelial cells (Moog, 1962). The appearance of alkaline phosphatase and epithelial cell differentiation are closely related to cell maturation. Staley (1969) has suggested that nonselective absorption of bacteria by the immature intestinal epithelial cell may be responsible for bacteremia and early

death in the newborn pig.

The Immune Response - Specific Factors Associated with Resistance

Excluding passive antibodies derived from the mother, normal hemolysins and agglutinins are absent from the blood of newborn infants and other neonatal animals (Wilson and Miles, 1964). The antibody response of the young animal varies greatly among species and antigenic stimulus. For example, specific antibodies have been demonstrated in sera of young infants with epidemic diarrhea due to pathogenic strains of Escherichia coli (Ibid.). Stimulation of 3-week-old pigs with E. coli and Salmonella pullorum antigens resulted in the formation of specific serum antibodies (Miller, et al., 1962; Sharpe, 1965; Miniats and Ingram, 1967). However, the protective effect of circulating E. coli antibody in swine is questionable (Miniats and Ingram, 1967). Lecce and Reep (1962) suggested that resistance of 2-week-old pigs to E. coli 08, which was pathogenic to newborn pigs, was not dependent on serum antibody.

Correlation between coproantibody and protection against experimental cholera infection has been established in guinea pigs (Burrows and Havens, 1948; Burrows, Elliot, and Havens, 1947); but, guinea pigs which had high serum titers were not necessarily resistant to oral infection in the absence of coproantibody.

It has been established recently that IgA is the pre-

dominant immunoglobulin in the lymphoid cells and secretions of the human gut (Crabbe and Heremans, 1966; Gelzayd, Kraft and Kirsner, 1968; Tomasi, 1968). Secretory IgA appears to be a dimer of serum IgA, plus a glyocoprotein T component or secretory piece (Watson, 1969). The secretory piece is believed to be synthesized in the epithelial cell while the IgA molecule originates in local lymphoid cells (Cebra, 1969; Tomasi, et al., 1965). Further evidence of a local immune system is given by Ogra and Karzon (1969).

Studies of IgA from porcine colostrum reveal much variation in the size of the IgA molecule, ranging from the molecular weight of serum IgA up to milk secretory IgA (Porter, Noakes and Allen, 1970). This group of investigators concluded that both serum and mammary gland may contribute to form the IgA that appears early in porcine colostrum. The precise role of secretory IgA is uncertain although antibacterial properties have been reported in human and porcine colostrum (Adinolfi, et al., 1966; Porter, Noakes and Allen, 1970).

The Role of Escherichia coli in Neonatal Disease

Escherichia coli normally inhabits the bowel of man and animals soon after birth and generally is considered to contribute to their well-being (Taylor, 1966). The pathogenic properties of E. coli became apparent after certain E. coli strains were recovered consistently from cases of infantile diarrhea in which no recognized pathogens could be

found (Edwards and Ewing, 1962). Bray (1945) and Bray and Beavan (1948) further emphasized the role of enteropathogenic E. coli by recovering the same serotype from 42 of 44 cases of infantile diarrhea. It was soon apparent that several serologic types of E. coli were associated with diarrheal diseases of the young child (Edwards and Ewing, 1962).

E. coli diseases in swine were also found to be associated with specific E. coli serotypes (Sojka, Lloyd and Sweeney, 1960). Some E. coli serotypes are primarily systemic pathogens while others are enteric pathogens, and certain serotypes are linked to distinct pathologic conditions (Ibid.).

The importance of predisposing factors and other intestinal flora is well recognized (Ibid.). However, the role of E. coli as an etiologic agent in diarrheal disease has been emphasized by the reproduction of clinical colibacillary diarrhea in gnotobiotic pigs, in the absence of environmental stress and other infectious agents (Kohler and Bohl, 1966a). Field surveys in England indicate that E. coli is the primary agent associated with diseases in pigs less than 3-weeks old (Anon., 1959, 1960).

Diseases Associated With Escherichia coli Infection

Several diseases have been attributed to Escherichia coli infections (Sojka, 1965; Barnum, Glantz and Moon, 1967). E. coli infections may result in colisepticemia, a condition

which commonly occurs in neonatal calves and pigs (Barnum, Glantz, Moon, 1967). The disease usually follows an acute fatal course, and death may occur without clinical manifestations (Ibid.).

Sojka (1965) reports that neonatal colibacillary diarrhea is another form of E. coli infection in pigs. In this condition diarrhea appears shortly after birth, and results in severe weakness and dehydration. Recovery or death may occur several days after the appearance of diarrhea. Enteropathogenic E. coli usually can be isolated in pure culture from the intestinal tract of diseased pigs (Ibid.).

Colibacillary diarrhea also may occur in thriving pigs at 3 to 6 weeks of age (Ibid.). Clinical symptoms include fever, diarrhea, dehydration, emaciation and retarded growth rate in severely affected surviving individuals (Ibid.; Barnum, Glantz, and Moon, 1967).

Pathogenic Properties of Escherichia coli

It is generally agreed that Escherichia coli-induced diseases result from proliferation of ingested organisms in the gut. Some strains may invade tissue and enter the blood from the intestinal tract (Barnum, Glantz, and Moon, 1967). Invasive E. coli strains are capable of inducing enteritis in neonatal pigs and may be isolated from internal organs during the course of the disease (Sojka, Lloyd, and Sweeney, 1960). Invasion, however, is not essential for the pro-

duction of colibacillary diarrhea (Barnum, Glantz and Moon, 1967). Smith and Jones (1963) did not observe tissue invasion in natural cases of colibacillary diarrhea, but concluded that the organism must adhere to and proliferate in the anterior small intestine in order to cause disease.

Recent studies, utilizing the ligated intestinal loop, indicate that the ability of some E. coli strains to produce diarrhea in animals is dependent on production of an enterotoxin (Smith and Halls, 1967a). Tests on living suspensions of enteropathogenic and nonenteropathogenic strains resulted in fluid accumulation and subsequent dilatation of ligated loops by enteropathogenic strains, whereas, nonenteropathogens did not induce loop dilatation. Tests on cell-free extracts of these same strains yielded similar results (Smith and Halls, 1967b). Additional evidence of enterotoxin production by certain E. coli strains has been presented (Smith and Halls, 1967a). Injection of ligated loops with strains of E. coli isolated from diseased pigs resulted in dilatation, but total numbers of E. coli in these segments were no greater than the numbers in undilated loops, which were injected with E. coli isolated from healthy pigs. Therefore, it is probable that factors other than mere proliferation are responsible for the pathogenic action of certain E. coli strains (Ibid.).

CHAPTER III

MATERIALS AND METHODS

Design

Two treatments were assigned both to newborn and to 3-week-old gnotobiotic pigs. Treatment I consisted of 17 newborn and nine 3-week-old pigs, which were infected orally with Escherichia coli 055B5. Pigs in Treatment I were euthanitized at selected intervals, and degree of E. coli invasion and migration was studied in the newborn and 3-week-old pigs. In Treatment II 25 newborn and eighteen 3-week-old gnotobiotic pigs were infected with E. coli 055B5. The resulting clinical syndromes were observed during the subsequent 6 day period. Noninfected gnotobiotic pigs were similarly studied for control purposes for each group.

Experimental Animals

Gravid sows of Hampshire, Yorkshire or Poland China breeding were obtained from Oklahoma State University farms or surrounding private swine farms. All pigs were delivered by standard or germfree hysterotomy at 112 to 115 days gestation. Sow anethesia was induced and maintained by the use of a halothane oxygen mixture in a circle system using

a partial rebreathing technique. No premedicants were used. Germfree pig procurement was carried out following the general recommendations of Landy, Growdon and Sandberg, (1961), Meyer, et al., (1963), and Trexler (1963). Newborn pigs in Treatment I were maintained in sterile cardboard isolators. All other pigs were transferred to stainless steel rearing isolators where they were maintained by methods of Meyer, et al., (1963) for the duration of the experiment. Pigs were fed a synthetic milk diet.[1]

Sterility Control of Gnotobiotic Systems

Isolator contents (flooring, excreta, pigs' feces and feet) were cultured for bacteria each time materials were introduced and/or removed from the isolators. Culture media, which included blood agar, Endo Agar[2] and Thioglycollate[3] medium, were inoculated in duplicate and incubated at room temperature and 37°C. Cultures were observed for 2 weeks before discarding negatives. Gram stains were made from the original swabs and examined for bacteria.

Propagation and Preparation of Challenge

The stock culture of Escherichia coli 055B5 was maintained on infusion agar slants in the dark at room temperature. Flasks containing 12 to 14 liters of nutrient

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[1]Baker's Liquid Milk, J. B. Roering Division, Chas. Pfizer New York, N. Y.

[2,3]Difco Lab., Detroit, Mich.

broth were inoculated with 8-hour E. coli broth cultures. Sterile air was bubbled through the medium during the 18 to 24 hours 37°C incubation period. Bacteria were harvested by centrifugation, and the concentrated bacteria washed 3 times with sterile saline. After the third wash the supernatant was discarded and the packed bacteria cells weighed. A 10% suspension was made by adding the proper amount of sterile saline. Plate counts on the dose suspension revealed approximately 1 X 10^9 bacteria per ml.

Collection of Tissue

Pigs were anesthetized with sodium pentobarbital,[4] scrubbed with surgical soap and the surgical area painted with 7% tincture of iodine. Tissues for bacterial cultures were removed by electrocautery to prevent contamination of adjacent tissue during excision. Liver and spleen were collected, and mesenteric lymph nodes draining the duodenum, jejunum and ileum were excised. Swabs of intestinal contents were streaked onto Endo Agar.

Intestinal segments for histologic studies were removed from the following locations: (a) 3 inches proximal to ileocecal valve (b) 15 inches proximal to ileocecal valve and (c) the site at which the duodenum passes beneath ascending colon. Tissues for fluorescent antibody studies were quick-frozen in liquid nitrogen and stored at -20°C

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[4]"Diabutal" Diamond Laboratories, Des Moines, Iowa

until studied. Formalin-fixed tissue was embedded in low-temperature-melting paraffin and stored at 4°C. Four micron sections were cut and stained with hematoxylin and eosin, Gram's stain and fluorescent antibody.

Fluorescent Antibody Techniques

A crude globulin fraction was obtained by fractionating immune rabbit serum with 45% ammonium sulfate and refrigerated overnight. The precipitate was recovered by centrifugation. Excess ammonium sulfate was removed by dialyzing against cold 0.85% NaCl. The protein content globulin fraction was determined by means of refractometer.[5] The globulin was labeled with fluorescein isothiocyanate[6] using the method described by Cherry, et al., (1960). The labeled globulin was divided into 2 ml. aliquots and stored at -20°C. The 2 ml. aliquots of conjugate were removed as needed and diluted with porcine liver and intestinal homogenate to the highest point which resulted in a 4+ stain reaction (usually 1/40 to 1/80).

Paraffin sections to be stained with fluorescent antibody were treated as follows: (1) immersed in 2 changes of xylene for 6 and 1 minutes respectively (2) placed in acetone for less than one minute and (3) carried through an alcohol series as follows: absolute ethanol, 95% ethanol,

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[5]Baush and Lomb, Rochester, N. Y.

[6]Baltimore Biological Lab., Baltimore, Md.

80% ethanol and 2 changes of distilled water for one minute each. (If sections appeared cloudy they were returned to the acetone and the procedure repeated from that point.) (4) applied conjugate and incubated sections in a moist chamber at room temperature for 30 minutes. (5) stained with conjugate and placed in phosphate buffered saline (pH 7.6) for 10 minutes and rinsed in distilled water. (6) mounted while still wet in pH 7.6 buffered glycerol.

Fluorescent antibody preparations were examined by means of a Standard GFL Zeiss microscope with a dark field condenser and an Osram HBO 200 Super Pressure Mercury light source. The ultraviolet excitation filter used was UG5 in combination with barrier filters 65/47.

Tube Agglutination

Pigs were bled from the anterior vena cava and the blood allowed to clot at room temperature. Serum and cells were separated by centrifugation. The serum was removed and stored at -20°C. Agglutination tests were carried out according to the method used by Cambell, et al., (1964).

CHAPTER IV

RESULTS

Migration and Invasion of *Escherichia coli* in Newborn Gnotobiotic Pigs

Two Hours after Exposure

Five of 6 pigs yielded positive cultures from duodenal lymph nodes two hours after intragastric exposure to *Escherichia coli* (Table I). Jejunal lymph nodes were positive in 4 of 6 pigs while none of the 6 ileal nodes cultures was positive. Bacteria were recovered from all 6 spleens and from 1 of 6 livers.

Six Hours after Exposure

Duodenal lymph node cultures taken 6 hours after exposure to *E. coli* were negative, but positive cultures were obtained from jejunal lymph nodes of 3 of 5 pigs and from ileal nodes of 2 of 5 pigs. Positive cultures were obtained from spleens of 5 pigs but from none of 5 livers.

Twenty Hours after Exposure

Cultures taken at 20 hours after exposure to *E. coli* revealed the presence of bacteria in duodenal lymph nodes

TABLE I

BACTERIOLOGIC RESULTS FROM NEWBORN GNOTOBIOTIC PIGS SUBSEQUENT TO INTRAGASTRIC EXPOSURE TO *E. COLI*

INTERVAL AFTER EXPOSURE	Duod[a]	Jej[b]	Ileum[c]	Spleen	Liver	Intestinal Lumen
2 HOURS	5/6[d]	4/6	0/6	5/6	1/6	5/6
6 HOURS	0/5	3/5	2/5	5/5	0/5	5/5
20 HOURS	3/6	6/6	6/6	4/4	3/6	5/5
0 HOURS CONTROLS	0/5	0/5	0/5	0/5	0/5	0/5
20 HOURS CONTROLS	0/2	0/2	0/2	0/2	0/2	1/2

[a]Lymph nodes draining the duodenal region of the small intestine.

[b]Lymph nodes draining the jejunal region of the small intestine.

[c]Lymph nodes draining the ileal region of the small intestine.

[d]Number of pigs in which cultures were positive/number of pigs cultured.

of 3 of 6 pigs. Bacteria were recovered from jejunal and ileal lymph nodes of 6 pigs. Four of 6 spleens and 3 of 6 livers were positive 20 hours after exposure. Samples from unexposed control pigs yielded negative cultures.

Light Microscopy

Six micron paraffin sections differentiated with the aid of Gram's stain were examined in order to characterize the invasion and migration of E. coli from the lumen to the mucosa, and submucosa. Relatively few bacteria were found in the lumen of the duodenum 2 hours after exposure. Microorganisms were present in great numbers in the lumen of the jejunum and ileum. Bacteria occasionally were attached to the epithelial cells lining the upper ½ of the villi of both jejunum and ileum. Six hours following oral exposure to E. coli few bacteria were observed in the lumen of either the duodenum or jejunum, but bacteria were abundant in the lumen of the ileum; a few bacteria were attached to the mucosal surface of the latter. Twenty hours after exposure bacteria were rarely observed in the duodenum and seen only occasionally in the jejunum. Numerous bacteria were present in the lumen of the ileum, and attachment to epithelial cells occurred at the base and apex of the villi. The attachment sites were usually one of 3 types. (1) sites which were characterized by aggregates of bacteria trapped in the folds of villi (2) sites which were associated with goblet and adjacent epithelial cells (3) sites involving 2 to 4

epithelial cells near the apices of the villi.

Migration and Invasion of *Escherichia coli* in 3-Week-Old Gnotobiotic Pigs

Two Hours after Exposure

Two hours after exposure positive bacterial cultures were obtained from duodenal and jejunal lymph nodes in 2 of 3 pigs, and from ileal nodes in 3 of 3 pigs (Table II). Bacteria were recovered from 0 of 3 spleens and from 0 of 3 livers collected from pigs 2 hours after exposure to *Escherichia coli*.

Six Hours after Exposure

One 3-week-old pig was euthanitized 6 hours after exposure; duodenal and ileal lymph nodes were positive, whereas, jejunal lymph nodes, liver and spleen were negative.

Twenty Hours after Exposure

Bacteria were recovered from duodenal, jejunal and ileal lymph nodes in 5 of 5 pigs euthanitized 20 hours after exposure; two of 5 spleens and 0 of 5 livers yielded positive cultures. Cultures from unexposed controls were negative.

Light Microscopy

Observations of Gram-stained sections revealed numerous luminal bacteria primarily in the duodenum, but also in the

TABLE II

BACTERIOLOGIC RESULTS FROM THREE-WEEK-OLD PIGS SUBSEQUENT TO INTRAGASTRIC EXPOSURE TO *E. COLI*

INTERVAL AFTER EXPOSURE	Duod[a]	Jej[b]	Ileum[c]	Spleen	Liver	Intestinal Lumen
2 HOURS	2/3[d]	2/3	3/3	0/3	0/3	3/3
6 HOURS	1/1	0/1	1/1	0/1	0/1	0/1
20 HOURS	5/5	5/5	5/5	2/5	0/5	5/5
20 HOURS CONTROLS	0/3	0/3	0/3	0/3	0/3	0/3

[a]Lymph nodes draining duodenal region of the small intestine.

[b]Lymph nodes draining jejunal region of the small intestine.

[c]Lymph nodes draining ileal region of the small intestine.

[d]Number of pigs in which cultures were positive/number of pigs cultured.

jejunum and ileum 2 hours after exposure to E. coli. Bacteria occasionally were observed adhering to epithelial cells, but were not detected penetrating the epithelium. Six hours after exposure organisms were still predominantly in the lumen of the proximal small intestine. By 20 hours after exposure bacteria were seen in the lumen of the duodenum and jejunum only occasionally, but they were more numerous in the lumen of the ileum. Bacterial attachment to epithelial cells was common. Bacteria were not detected in the epithelial cell as described in the newborn pig. Penetration of epithelium was apparent but was limited to the luminal border. In no instance were bacteria observed basal to the nucleus of the epithelial cell.

Pathogenicity of Escherichia coli to Newborn Gnotobiotic Pigs

Before presenting the results of this experiment, the term diarrhea should be defined. Diarrhea as used in this report indicates a range of fecal type from those of frequently excreted yellow liquid to that of yellow and pasty in consistency.

After exposing newborn pigs to Escherichia coli, 9 of 25 died in less than 24 hours without previous signs of disease (Table III). Eleven of 25 E. coli-exposed pigs had typical colibacillary diarrhea and died at intervals ranging from 36 to 120 hours. Five exposed pigs which had colibacillary diarrhea, recovered after 3 to 4 days. Seven

TABLE III

MORTALITY OF NEWBORN GNOTOBIOTIC PIGS SUBSEQUENT TO INTRAGASTRIC EXPOSURE TO *E. COLI*

HOURS AFTER EXPOSURE	12	18	24	36	48	60	72	120	144
NO. PIGS EXPOSED	25	--	--	--	--	--	--	---	---
CUMULATIVE NO. OF FATALITIES	2	3	9	11	13	15	19	20	20
% MORTALITY	8	12	36	44	52	60	76	80	80

All (7) control pigs remained healthy and were euthanitized when 6 days old as were 5 surviving pigs.

unexposed pigs remained healthy.

Diarrhea was observed in newborn pigs 18 to 24 hours after exposure to E. coli. Dehydration, weakness, straining to defecate and general depression were evident in exposed pigs although severity of disease varied among pigs. Pyrexia (2-4° above normal) and inappetance accompanied the diarrhea. Fever usually persisted for 4 to 6 hours at which time death sometimes occurred. Comatose pigs were removed from isolators, anesthetized, and tissue was taken for histologic study. Pigs were euthanitized immediately after samples were taken. Postmortem findings did not reflect the severity of the disease. The small intestine was sometimes distended, flaccid, and occasionally petechiae were present. The stomach of some pigs contained chunks of partially digested milk; intestinal contents were usually watery and yellow in color. Extensive lesions were not observed.

Examination of Gram-stained paraffin sections revealed apparently random bacterial attachment over the entire villus, and organisms had entered the epithelial cells (Figures 1 through 4). Four or more epithelial cells were usually involved at any one penetration site. Intracellular organisms were observed in various stages of migration throughout the epithelial cells (Figures 5, 6). Penetration was not observed in the crypt cells.

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LEGENDS FOR FIGURES

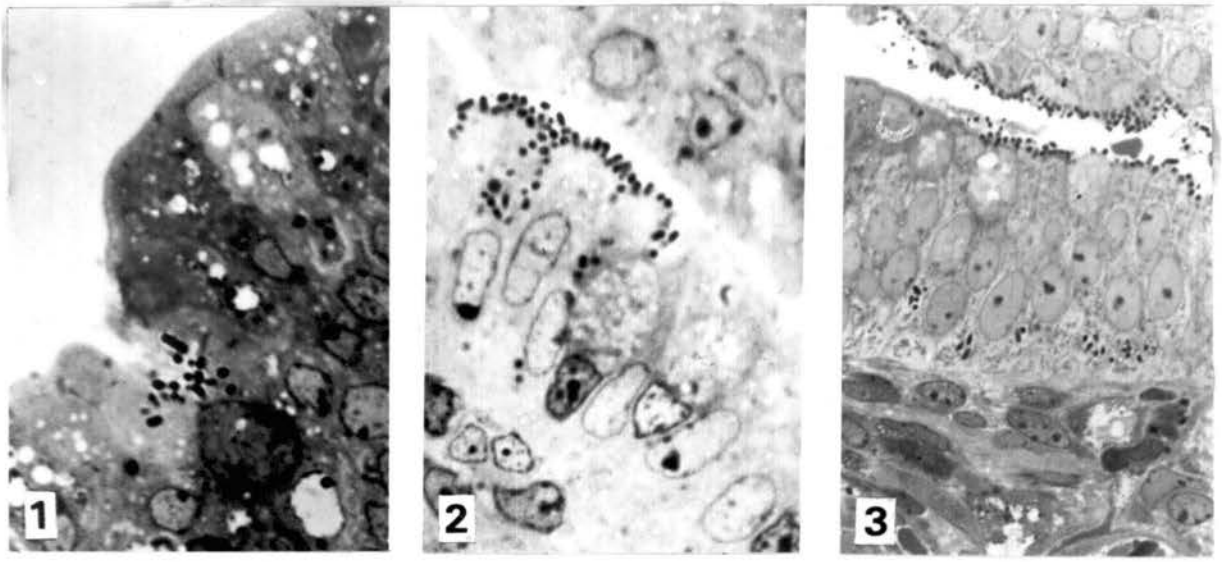
All micrographs are of the ileums of neonatal or 3-week-old gnotobiotic pigs exposed to *Escherichia coli*. Figures 1 through 3 are 1-micron thick sections of Epon-Araldite-embedded tissue stained with 1% toluidine blue. Figure 4 is an electron micrograph stained with lead citrate and uranyl acetate.

Figure 1. Intestinal epithelium of neonatal pig ileum 20 hours after exposure to *E. coli* (mid-villous region). *E. coli* have collected on and penetrated the epithelium in a fold of mucosa near the mouth of a goblet cell. X1000

Figure 2. Intestinal mucosa of neonatal pig ileum 20 hours after exposure to *E. coli* (mid-villous region). *E. coli* are attached to the surface of absorptive cells and have penetrated cytoplasm. X1000

Figure 3. Intestinal absorptive cells of neonatal pig ileum 20 hours after exposure to *E. coli* (upper 1/3 of villus). Microorganisms are attached to the mucosal surface and are present in the subnuclear region of the epithelial cells. X1000

Figure 4. Epithelial cells of neonatal pig ileum 20 hours after exposure to *E. coli*. Bacteria have exfoliated the microvillous border and have attached to the surface of the plasma membrane. Organisms are also in membrane-bound vacuoles below the nuclei. X4000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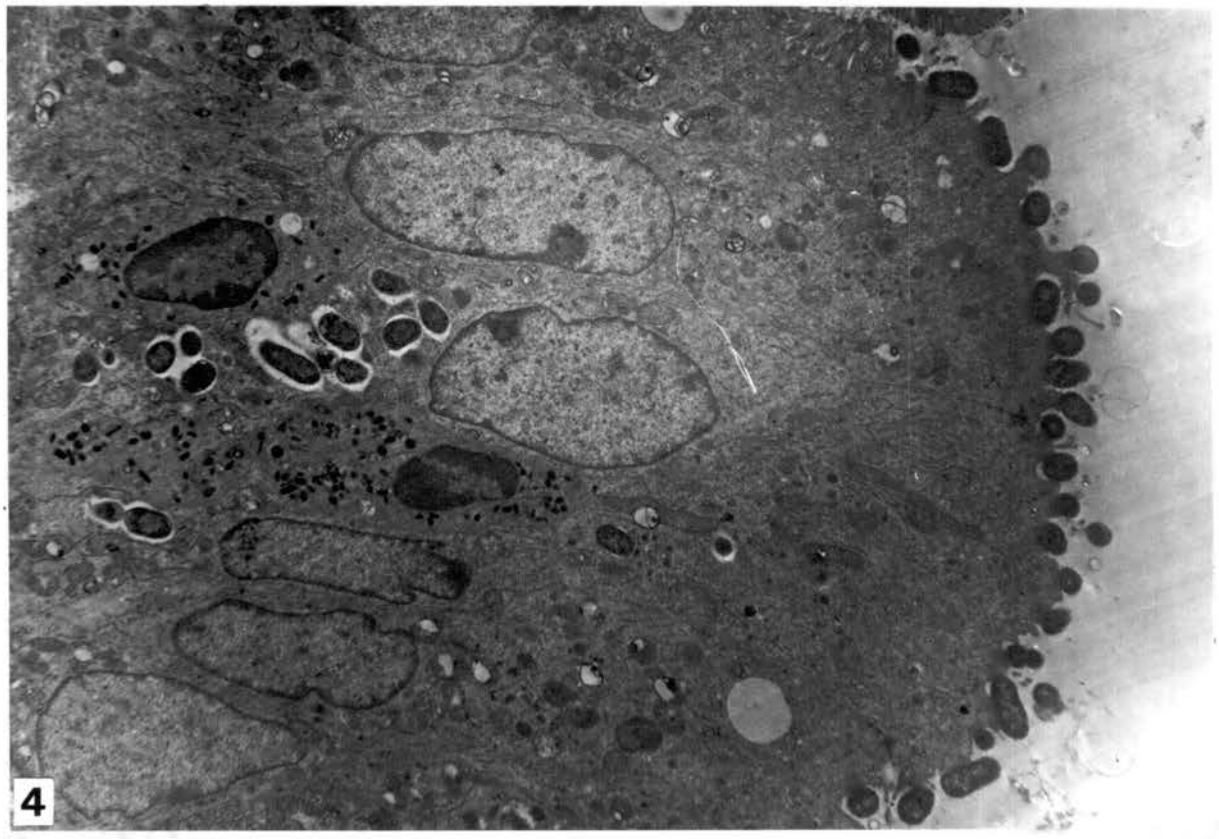
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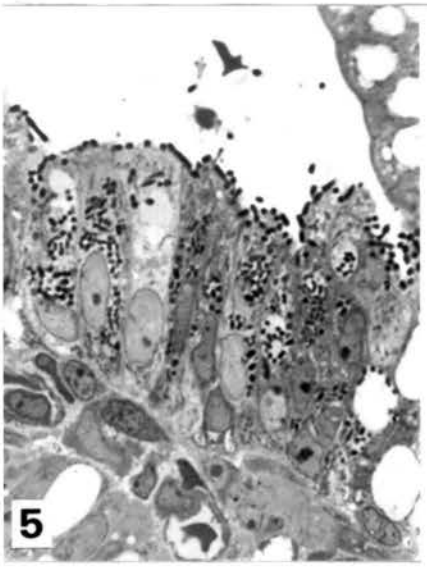
LEGENDS FOR FIGURES

Micrographs are of ileums of neonatal pigs exposed to Escherichia coli. Figure 5 is a 1-micron thick section embedded in Epon-Araldite and stained with 1% toluidine blue. Figure 6 is an electron micrograph stained with lead citrate and uranyl acetate.

Figure 5. Absorptive cells on lower villus (48 hour E. coli-exposed pig). E. coli are attached to and invading the epithelial cells. X1000

Figure 6. Epithelial cells 60 hours after exposure to E. coli. Bacteria have exfoliated microvillous border and are present throughout the epithelial cells. X6000



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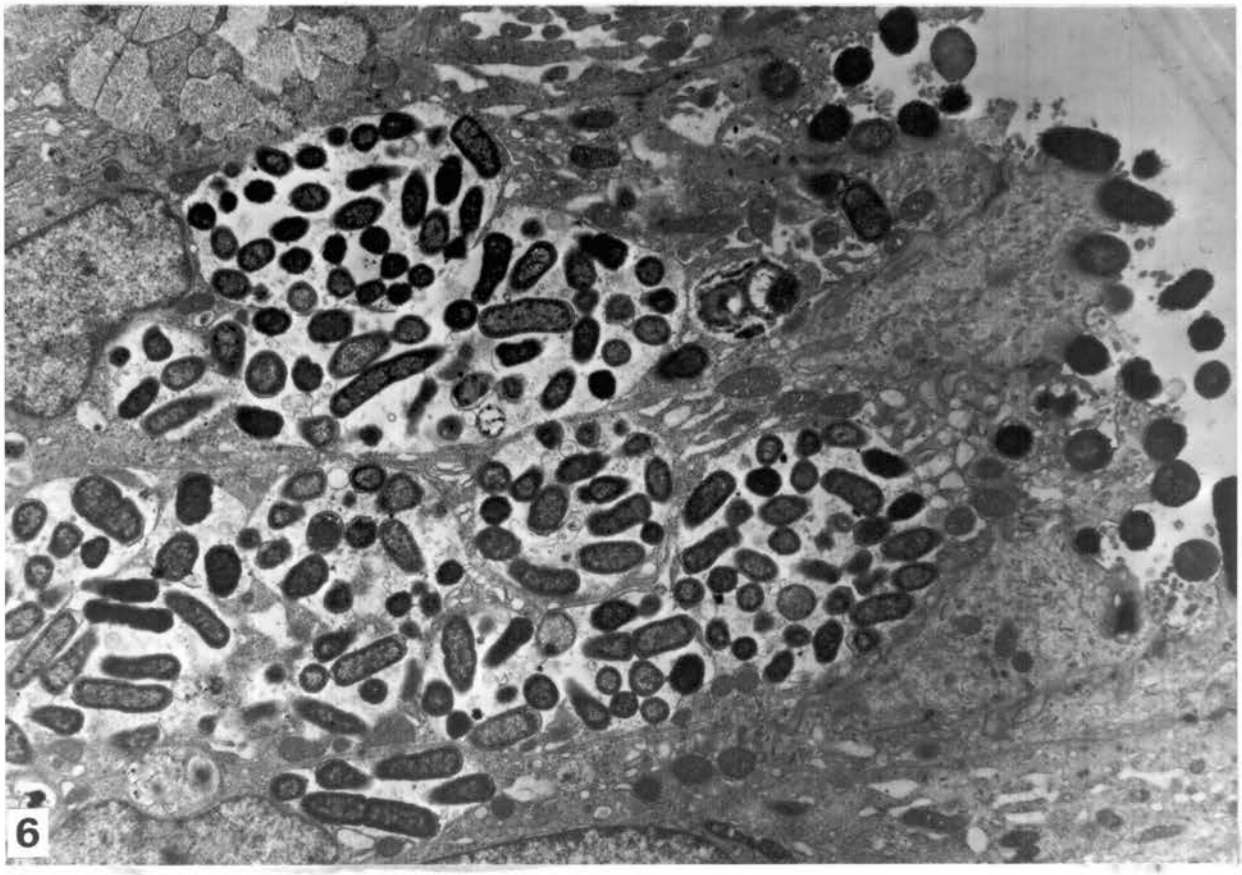

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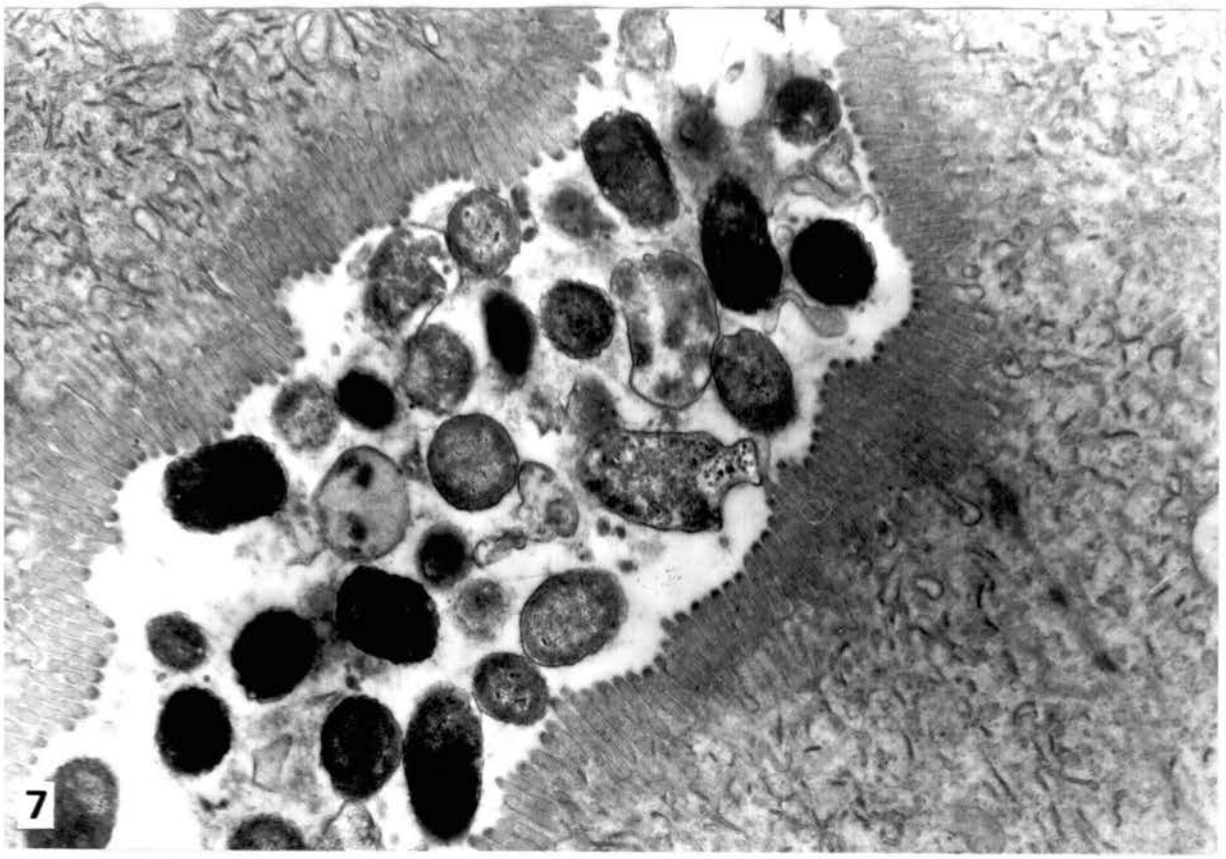
LEGENDS FOR FIGURES

Figures 7 and 8 are electron micrographs of neonatal pig ileum exposed to *Escherichia coli*. Sections are stained with lead citrate and uranyl acetate.

Figure 7. Apical ends of epithelial cells 6 hours after exposure to *E. coli*. The microvillous border is intact; numerous organisms are present in the intestinal lumen. X12,500

Figure 8. Apical ends of epithelial cells 20 hours after exposure. Fimbriated *E. coli* are attached to the surface of the plasma membrane. X18,000



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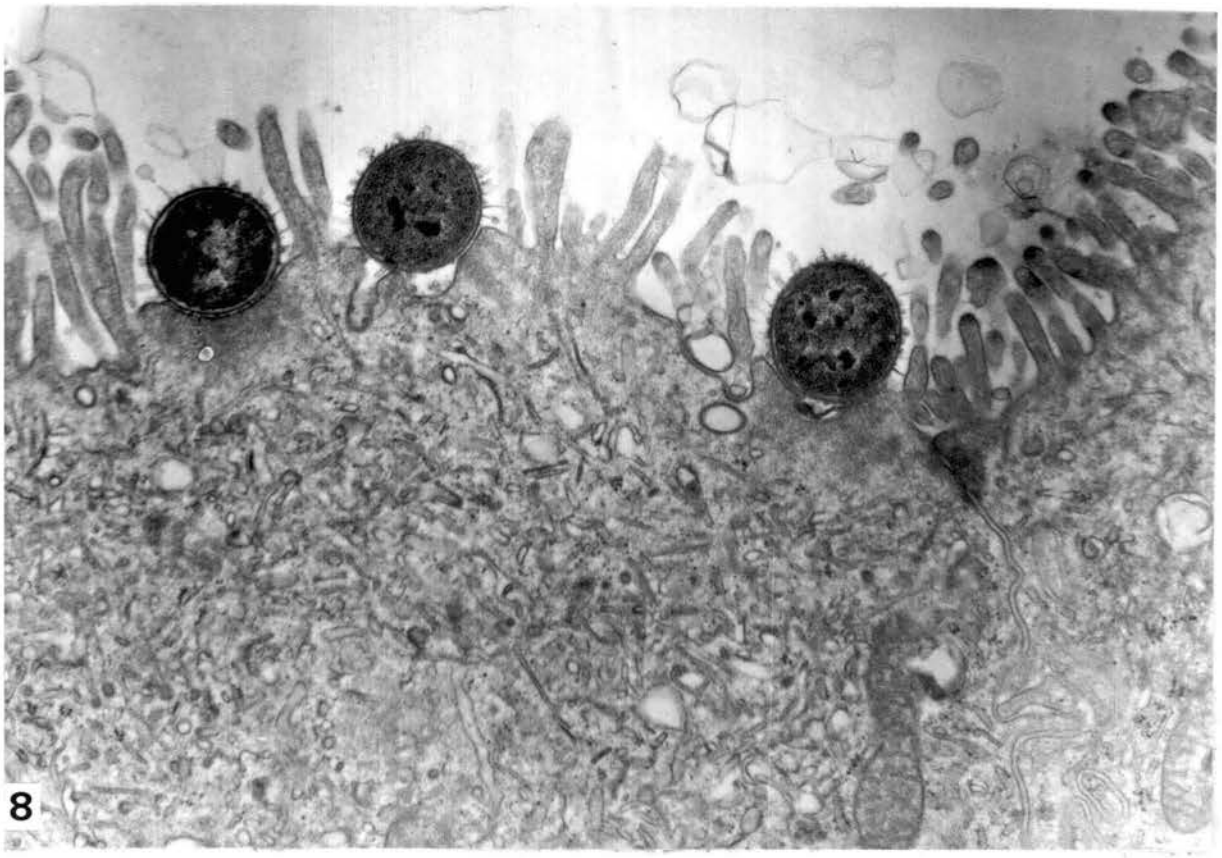

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Pathogenicity of *Escherichia coli* to 3-Week-Old Gnotobiotic Pigs

Eighteen gnotobiotic pigs were exposed to *E. coli* at 3 weeks of age. Two pigs died within 24 hours after exposure with signs of only slight depression; diarrhea was not observed. Two pigs which developed colibacillary diarrhea were euthanitized *in extremis* at 60 and 72 hours following exposure to *E. coli* (Table IV).

All exposed pigs eventually developed colibacillary diarrhea. In some pigs diarrhea commenced 16 hours after exposure, while in others diarrhea was not observed until 24 to 36 hours. In some instances transient fever (104° to 106°F), slight depression and inappetance accompanied the diarrhea; however, these signs usually did not persist beyond 64 to 72 hours after exposure. Other pigs which had diarrhea remained alert and vigorous throughout the 6 day period. Blood cultures from the pig euthanitized 60 hours after exposure yielded 6×10^5 bacteria per ml. of blood. Bacteria (2×10^2 ml.) were recovered from the blood of one surviving pig. All other blood cultures were negative. Tube agglutination tests revealed antibody titers (40 to 640) to homologous "K" antigens (Table V).

Examination of Gram-stained tissue sections from the duodenum, jejunum and ileum of pigs exposed to *E. coli* at 3 weeks of age revealed few invading organisms.

A few bacteria were present in the lumen of the jejunum,

TABLE IV

MORTALITY OF THREE-WEEK-OLD GNOTOBIOTIC PIGS SUBSEQUENT TO INTRAGASTRIC EXPOSURE TO *E. COLI*

HOURS AFTER EXPOSURE	12	18	24	36	48	60	72	120	144
NO. EXPOSED	18	--	--	--	--	--	--	---	---
CUMULATIVE NO. OF FATALITIES	0	0	2	2	2	2	3	4	4
% MORTALITY	0	0	11	11	11	11	17	22	22
MORBIDITY	--	--	--	100%	--	--	--	---	---

TABLE V

"K" ANTIBODY TITERS FOUND IN THE SERUM OF GNOTOBIOTIC PIGS EXPOSED TO E. COLI AT 3 WEEKS OF AGE[a]

PIG NO.	Serum Dilution 1/20	1/40	1/80	1/160	1/320	1/640
35139[b]	-	-	-	-	-	-
38159[b]	+	-	-	-	-	-
38167[b]	+	+	-	-	-	-
38166[b]	+	-	-	-	-	-
35140	+	+	+	-	-	-
35142	+	+	+	+	-	-
35143	+	+	+	+	+	-
35144	+	+	+	+	+	-
38168	+	+	+	+	+	+
38165	+	+	+	+	+	+
38156	+	+	+	+	+	+
38162	+	+	+	+	+	+
38163	+	+	+	+	+	+
38164	+	+	+	+	+	+

[a]Serum collected 6 days after E. coli exposure.

[b]Unexposed controls.

but large numbers of luminal bacteria were seen only in the ileum. Small clumps of bacteria were attached to the epithelial cells of the ileum, but attachment was sporadic and did not appear to disturb the microvilli (Figures 9 through 12).

LEGENDS FOR FIGURES

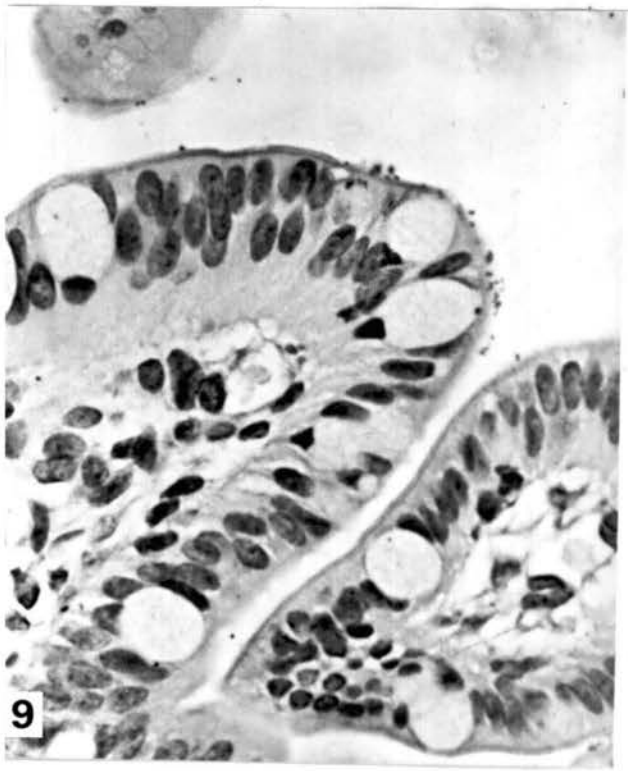
Figures 9 through 12 are micrographs of tissue from pigs exposed to Escherichia coli at 3 weeks of age. All sections are from 20-hour-exposed ileum. Six-micron paraffin sections are stained with Gram's stain.

Figure 9. Bacteria randomly attached to apex of villus. X400

Figure 10. Intestinal epithelial cells of upper 1/3 of villus. Small clump of bacteria near goblet cell. X1000

Figure 11. Intestinal epithelial cells near apex of villus. Numerous mucus-bound bacteria present in the lumen. X1000

Figure 12. Mucosa near apex of villus. Small clump of bacteria are attached to epithelium. X1000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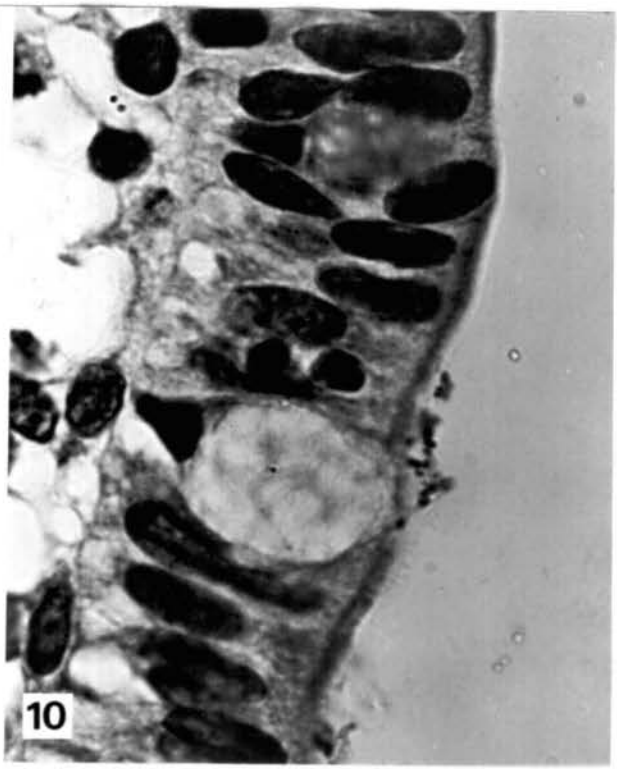
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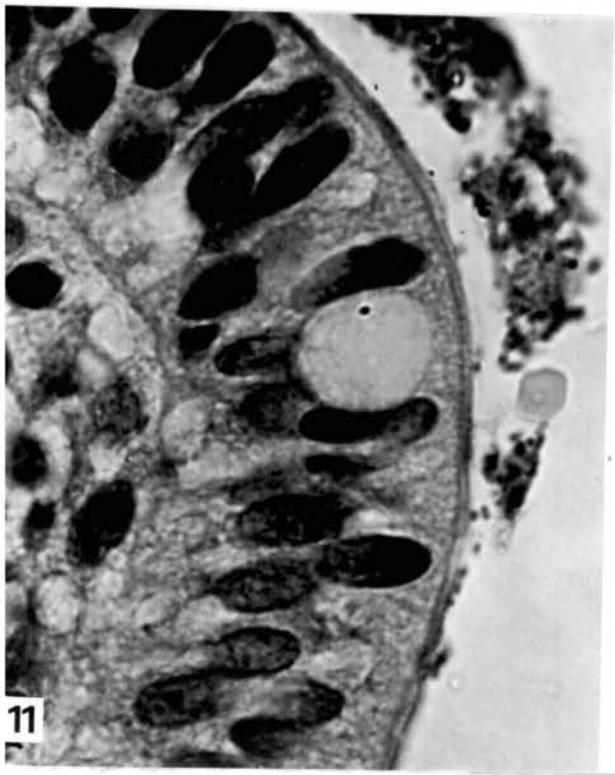
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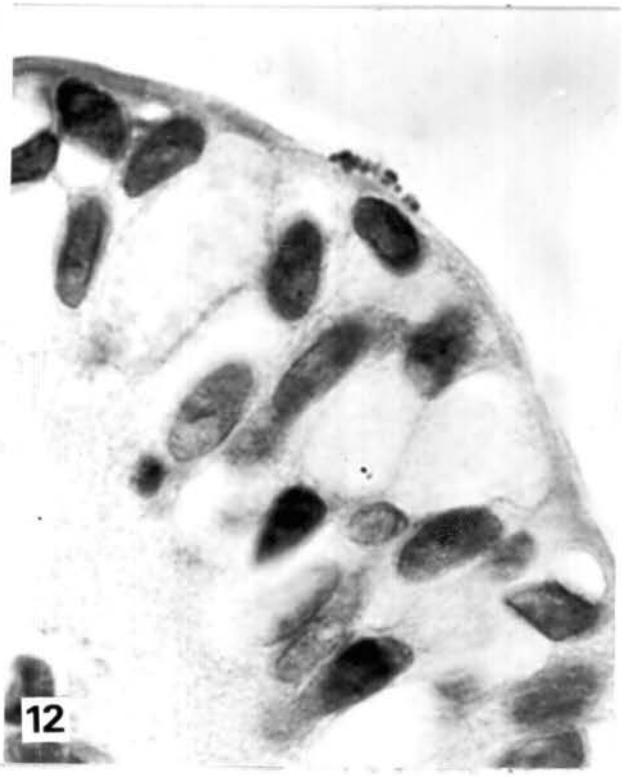
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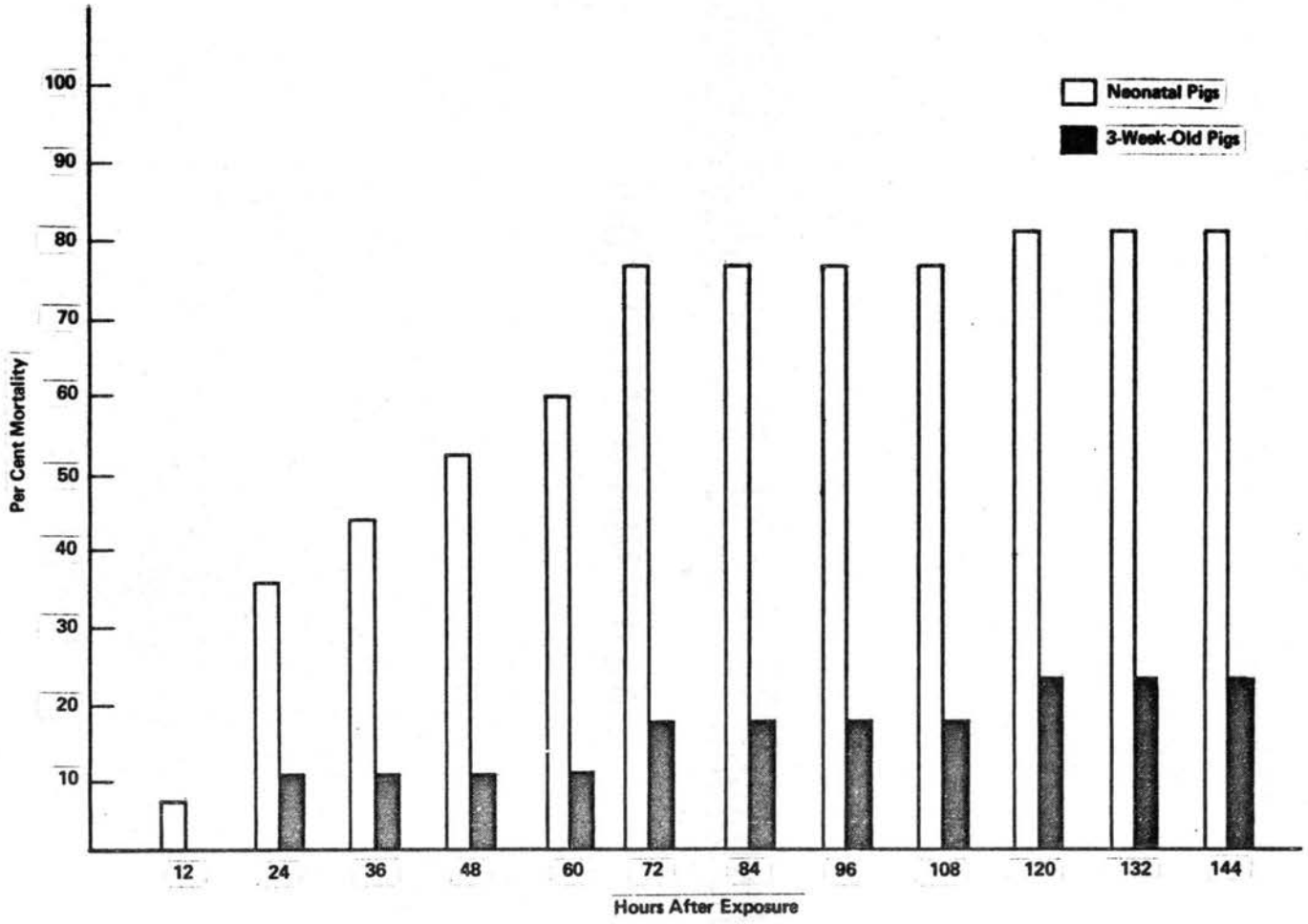

Figure 13. Mortality of Neonatal and 3-Week-Old Gnotobiotic Pigs after Exposure to E. coli

CHAPTER V

DISCUSSION

The results of this study indicate that resistance of the young pig to Escherichia coli infections develops during the first few weeks of life (Figure 13). It is well established that neonatal animals are susceptible to E. coli-induced diseases, but only recently have investigators been able to reproduce these diseases (Kohler and Bohl, 1966a; Kohler, 1967a; Kramer and Nderito, 1967; Kramer, 1968; Moon, Sorenson and Sautter, 1968). It appears that gnotobiotic or or hysterotomy-derived pigs are particularly susceptible to infection (Nielsen, Moon and Roe, 1968).

Resistance of the Neonate to Escherichia coli

Due to the passive transfer of maternal antibodies either through the placenta or by way of colostrum the neonatal animal generally is resistant to infectious diseases controlled by specific humoral antibody (Weiser, Myrvik, Pearsall, 1969). However, in diseases in which humoral antibody does not contribute to immunity, greatest susceptibility exists during the neonatal period (Ibid.) The young pig depends upon passively acquired colostral antibodies for protection from disease during the first 2 to 3

weeks of life, at which time signs of limited immune competence become apparent (Miller, et al., 1962; Sharpe, 1965).

The recovery of E. coli from duodenal and jejunal mesenteric lymph nodes 2 hours after administration of the challenge indicates that the bacteria rapidly penetrated the intestinal epithelium and migrated via lymphatic vessels. Positive cultures from 6 of 6 spleens and from 1 of 6 livers suggest that the microorganisms overwhelmed the first line of defense, the local lymphoid tissue, and were challenging secondary lymphoid elements.

Qualitative studies, by histologic and bacteriologic methods, denoted a predominance of bacteria in the caudal region of the small intestine of pigs euthanitized by 6 hours postexposure. This is indicative of a "flushing" effect of bacteria from proximal to distal small intestine, presumably due to peristaltic action of the intestine. Flushing of microorganisms by peristalsis has been shown to be an effective means of preventing Shigella invasion in adult guinea pigs (Formal, et al., 1964), whereas slowing of intestinal transit time can potentiate Salmonella infections (Sprinz, 1969). Alvarez (1940) reports that the rate of peristalsis is graded from approximately 22 contractions per minute in the duodenum to near 12 a minute in the terminal ileum. Koldovsky (1969) noticed an increase in intestinal motility of rats between 3 and 7 days of age, while Kidder, Manners and McCrea (1961) did not detect a change in movement along the intestinal tract when 6 and 17-

day-old pigs were compared.

Despite the fact that bacteria were recovered from lymph nodes at 2 to 6 hours after exposure, microorganisms were not detected in epithelial cells or underlying tissue, when Gram-stained serial tissue sections were examined by light microscopy. Staley, Jones and Corley (1969a) have suggested a relationship between colostrum absorption and bacterial penetration of intestinal epithelium in the neonatal pig.

At 20 hours postexposure bacteria were readily observed attached to and invading the intestinal epithelium of Gram-stained sections. The bacteria had a predilection for cells adjacent to goblet cells as described previously (Ibid.). Although organisms were observed invading epithelial cells adjacent to goblet cells, invasion of goblet cells themselves was not seen. In addition to these invasion sites, bacteria were commonly trapped in the folds of villi of the newborn pigs and subsequent invasion observed. These sites were usually midway between the crypts and apical regions of villi. Perhaps the entrappment of bacteria in the folds provides a niche for the bacteria to lodge and proliferate prior to invasion of the surrounding tissue. A third type and site of attachment was often seen. These sites usually involved 2 to 4 epithelial cells of the apical villi. Perhaps bacteria are mechanically pressed against the epithelium as a result of peristaltic action, or bacteria may cling to any surface with which they come into contact.

Fimbriae were observed on some of the attaching organisms. Fimbriae have been shown to be organs of attachment and possess hemagglutinating properties (Duguid, et al., 1955). Fimbriae, however, were not a consistent feature of attaching and invading organisms.

Bacteria were detected in various stages of migration throughout epithelial cells near attachment sites. E. coli could not be detected penetrating the basal membrane of epithelial cells of the newborn pig, although phagocytized organisms were demonstrated in cells of the lamina propria (Staley, Jones and Corley, 1969a). Bacteriologic studies confirmed the histologic observations on tissue exposed to E. coli for 20 hours. Positive cultures were obtained from 6 of 6 ileal lymph nodes. Recovery of bacteria from 4 of 6 spleens and 3 of 6 livers suggests that the organisms proliferate in regional lymph nodes and "spill over" into the general circulation. It is of interest that 6 of 6 jejunal nodes and 3 of 6 duodenal nodes were positive 20 hours after exposure to E. coli. This suggests repopulation of the anterior small intestine by E. coli. Previous investigations have revealed that proliferation of enteropathogenic E. coli in the proximal small intestine is necessary to produce neonatal colibacillary diarrhea (Kenworthy and Crabb, 1963; Smith and Jones, 1963; Neilsen, Moon and Roe, 1968). The data presented herein are in agreement with earlier investigations. At 18 to 20 hours after exposure of neonatal pigs to E. coli, typical manifestations of

colibacillary diarrhea were evident. The asymptomatic pigs which died suddenly within 24 hours postexposure presumably died of an acute septicemia. As mentioned earlier, tissues from these pigs were not cultured, but previous studies have revealed the presence of bacteria in peripheral blood 18 hours postexposure (Corley, unpublished data). Clinical observations of neonatal pigs which developed colibacillary diarrhea were consistent with those reported previously (Moon, Sorenson, and Sautter, 1968; Kohler and Bohl 1966a; Kohler 1967a). It is of interest that systemic invasion by E. coli was observed in this study as it was by Kohler (1967a); others, however, indicate that systemic invasion is not observed in colibacillary diarrhea (Moon, Sorenson and Sautter, 1968). Variation among strains of E. coli probably is responsible for these differences. Some strains of E. coli associated with diarrheal diseases can cause accumulation of large amounts of fluid and subsequent dilatation of ligated intestinal loops which, nevertheless, maintain an intact epithelium (Moon, Sorenson and Sautter, 1966; Smith and Halls, 1967a). The occurrence of transient neonatal colibacillary diarrhea and subsequent recovery of some infected pigs is not surprising. Variation in susceptibility among individual pigs, both gnotobiotic and conventional, is characteristic of E. coli-induced diseases (Kohler and Bohl, 1966a; Moon, Sorenson, Sautter, 1968).

Resistance of the 3-Week-Old Gnotobiotic Pig to *Escherichia coli*

The results of this investigation indicate that the 3-week-old gnotobiotic pig has developed limited resistance to *E. coli* during the first few weeks of life. This resistance is characterized by reduced mortality rate, lack of systemic involvement and a mild form of colibacillary diarrhea as compared to the infected newborn animal. Even though *E. coli* organisms passed beyond the intestinal epithelium of the 3-week-old pig they were arrested in the local lymphoid tissue and did not appear to enter the general circulation.

There is considerable disagreement concerning the development, role and efficiency of serum immunoglobulins in the prevention of *E. coli* induced diseases in swine. Upon oral infection with *E. coli*, Kohler (1966) demonstrated low levels (titer 4 to 32) of bactericidal and hemagglutinating activity in the serum of pigs vaccinated at 4 to 6 days of age. However, a subsequent intravenous injection was necessary to elicit an appreciable (500 to 1,000) titer in these animals. After failure to demonstrate significant levels of homologous serum agglutinins in these same pigs, Kohler (1966a) concluded that there was no correlation between *E. coli* serum agglutinins and the bactericidal-hemagglutinating activity in swine. Immunization of swine, ages 0 to 9 weeks, revealed that circulating *E. coli* anti-

body rendered little protection from E. coli diseases of the young pig (Miniats and Ingram, 1967). Contrasting results were obtained from naturally reared pigs; "K" antibodies were shown to be high on the second day of life and then to decrease until 3 weeks of age when a second rise became apparent (Sharpe, 1965). The initial high level presumably was due to absorption of colostral antibodies, whereas, the second rise was a result of the pig's own immune competence. Again, variation among strains of E. coli may be responsible for lack of agreement on the value of the circulating antibodies. Certain noninvasive E. coli strains /~0139:K83 (B)_7 tend to evoke antibody titers to somatic antigens. An invasive strain (08), which is frequently associated with colisepticemia and colibacillary diarrhea in neonatal pigs, induced high "K" antibody titers (Miniats and Ingram, 1967).

Experiments conducted in this study have shown that E. coli 055B5 penetrates the intestinal epithelium of 3 to 4-week-old gnotobiotic pigs and induces the formation of "K" antibodies. Serum from colostrum-deprived gnotobiotic pigs which were exposed to E. coli at 3 to 4 weeks of age, revealed the presence of specific "K" antibodies at the end of the 6 day experimental period (Table V). Only 10% of the pigs exposed at 3 weeks of age died of septicemia. However, "K" antibodies could not be detected in sera of 1 to 6-day-old pigs, and 35% of the pigs died of septicemia within 24 hours following exposure to E. coli. It is not

unreasonable to assume that circulating homologous "K" antibodies are instrumental in protecting the young pig from colisepticemia.

It has been suggested in this study that "K" antibodies may protect 3-week-old pigs from colisepticemia; other factors should be considered in the resistance of these pigs to colibacillary diarrhea. Lecce and Reep (1962) report that feeding 10^9 E. coli 08 caused disease in neonatal pigs but did not cause disease in 2-week-old colostrum-free pigs despite the absence of specific agglutinins. Factors to be considered are:

1) Maturation of the epithelial cell and associated enzyme systems
2) Increased turnover rate of epithelial cells in older pigs
3) Presence of local antibody

Staley (1969) indicates that few digestive enzymes are present during the early neonatal period and that absorption of macromolecular proteins by the intestine of the neonatal ungulate may result from immaturity of the intestinal epithelial cell. Furthermore, serum assays of neonatal pigs given large oral doses of protein revealed little proteolysis of absorbed protein (Pierce and Smith 1967). Payne and Marsh (1962a) report that an increase in the amount of alkaline phosphatase activity occurs in the small intestine of the baby pig at time of closure. The appearance of enzymes within the adult absorptive cell, as it migrates

from the crypt to villous tip, is an indication of maturity (Padykula, 1962). Further study of epithelial maturation as a mechanism of resistance seems appropriate.

The duration and nature of injury to the intestinal absorptive cell by enteropathogenic E. coli and/or metabolic products is unresolved. If the damage is permanent, acceleration of epithelial replacement would seem desirable. Little, however, is known concerning the relationship of epithelial replacement rate and the development of resistance in the young animal. Epithelial cell replacement rates are delayed in germfree animals but may be accelerated by protein absorption and exposure to bacteria (Abrams et al., 1963). Grey (1968) reports that epithelial cell migration from crypt to the apex of the villus is much slower in preweaned mice than in adult mice. Research on epithelial replacement in the pig is notably lacking. It tempts speculation that an age related increase in epithelial cell replacement may occur in the pig as it does in the mouse.

There is convincing evidence that specific antibody has protective properties within the intestinal lumen. Pigs 72 hours of age (a time at which pigs can no longer absorb intact proteins) were challenged with E. coli followed by oral administration of either normal[7] or hyperimmune homologous colostrum or serum. E. coli-exposed pigs which were

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[7]Low titer "natural" antibodies to E. coli were detected in the normal serum and colostrum.

not given either serum or colostrum died. Approximately 50% of the infected pigs which were fed either normal serum or colostrum survived, while all infected pigs given either hyperimmune serum or colostrum survived. Since antibodies could not be detected in the serum of surviving pigs, the protective function of antibodies administered orally was assumed to be directly in the intestinal tract (Travenicek, et al., 1967). Kohler and Bohl (1967a) found that homologous immune serum administered orally protected 4 to 6-day-old pigs infected with E. coli. However, diarrhea commenced 12 to 24 hours after administration of the last serum. Subsequent studies confirmed this report and revealed that at best, parenteral administration of immune serum provided only limited protection from colibacillary diarrhea in 4 to 6-day-old pigs (Kohler, 1967b). It is of interest that even though the pigs were refractory to diarrheal disease, oral administration of immune serum did not reduce viable bacterial counts in the intestine (Kohler, 1967b). Kohler and Bohl (1966b) concluded that the protective value of immune serum administered orally to pigs with colibacillary diarrhea is not dependent upon the conventional complement-requiring bactericidal activity.

Investigators present evidence indicating the value of local antibody in the prevention of E. coli-induced diarrhea in young pigs. This evidence implies that local antibody may be involved in producing resistance to colibacillary diarrhea in young pigs deprived of colostral antibody. It

has been proposed that local immunity and mucoantibodies may be of primary importance in the defense against some infections (Tomasi and Bienenstock, 1968).

IgA is the predominant immunoglobulin in intestinal fluids, colostrum, saliva and other external secretions which bathe mucous membranes in humans (Ibid.). The major fraction of IgA has a sedimentation coefficient of 11S and possesses a nonimmunoglobulin polypeptide chain referred to as the secretory piece or T component (Johnson, 1970). The T component of secretory IgA is believed to be responsible for its increased resistance to proteolytic digestion in the intestinal tract (Tomasi and Calvanico, 1968). IgA antibodies to E. coli have been demonstrated in human and porcine colostrum, although IgA is not the predominant immunoglobulin in porcine colostrum (Adinolfi, et al., 1965; Porter, Noakes and Allen, 1970). The mechanism by which secretory IgA antibody affords protection is not known. Adinolfi, et al., 1966, reported that colostrum IgA lysed bacteria in the presence of complement and lysozyme while other knowledge suggest IgA serves as an opsonin (Johnson, 1970). The latter seems to be the most acceptable explanation at this time.

The secretory immune system is believed to be distinct from the system responsible for producing circulating antibody (Tomasi and Bienenstock, 1968). Recent immunofluorescent studies on human salivary gland tissue, rabbit intestine, salivary glands, mammary glands and bronchi

indicate that secretory IgA is produced locally; the T component is synthetized in epithelial cells and the IgA in the underlying plasma cells (Cebra, 1969). Segmental immunization of distal colon with polio vaccine by means of a double-barrel surgical colostomy in humans resulted in the production of IgA antibodies only from the immunizied segment, whereas subjects immunized parenterally sometimes did not develop secretory antibody (Ogra and Karzon, 1969.; Johnson, 1970). This indicates that locally applied antigen stimulates local IgA production. Studies on the development of serum and salivary IgA systems in infants have contributed additional evidence that these are distinct systems (Haworth and Dilling, 1966). It is noteworthy that salivary IgA was detected consistently a few days before IgA was found in the serum (Ibid.). Salivary IgA was present in 4 of 7 premature infants 12 to 13 days after birth and in 5 of 9 full-term infants at 14 to 15 days of age (Ibid.). Furthermore, it was noted that salivary IgA was probably present at an earlier age than indicated by data collected because of dilution problems encountered during collection of samples (Ibid.).

The striking feature of pigs which were exposed at 3 weeks of age, when compared to neonatal pigs exposed to E. coli, was the relationship of E. coli to the intestinal mucosa. Attachment and invasion sites were not observed, and the relationship of bacteria to epithelial cells was casual. Large numbers of organisms were observed in the

intestinal lumen but appeared to be bound in luminal mucus (Figure 11). Admittedly, bacteria penetrated the intestinal epithelium, but tissue invasion by pathogens is not necessarily associated with disease (Sprinz, 1969).

Although direct experimental evidence concerning the role of secretory intestinal immunoglobulins was not presented in the present study, the possibility exists that intestinal immunoglobulins may be primarily responsible for the resistance of the 3-week-old gnotobiotic pig to colibacillary diarrhea. The role and development of intestinal immunoglobulins in the pig have not been defined, and additional research in this area is worthy of consideration.

Summary and Conclusions

Neonatal and 3-week-old gnotobiotic pigs were given an intragastric challenge of Escherichi coli 055B5. Pathogenic properties of the microorganism and resistance of the host were studied by microbiologic and histologic methods.

Neonatal pigs were found to be quite susceptible to colisepticemia and colibacillary diarrhea as evidenced by an 80% mortality rate. Bacteriologic studies revealed that E. coli penetrated the epithelium of newborn pigs 2 hours after exposure, although penetration could not be detected by histologic methods until 20 hours after exposure. Gnotobiotic pigs challenged at 3 weeks of age were somewhat more refractory to E. coli infection, having a mortality rate of only 20%.

Neonatal colostrum-deprived pigs had not produced detectable homologous circulating antibodies when tested at 1 to 6 days after exposure to E. coli. These pigs seemed particularly susceptible to colisepticemia. Gnotobiotic pigs which were exposed to E. coli 3 weeks after birth had appreciable titers 3 to 6 days later; few of these pigs died of septicemic cause. These results suggest that homologous antibodies circulating in sera of 3 to 4-week-old pigs may be responsible for their resistance to colisepticemia.

Exposure of 3-week-old pigs to E. coli resulted in much milder diarrhea than that experienced by their neonatal counterparts. Evidence was not presented to elucidate the differentiating factors. It is hypothesized that development of a local immune system during the first few weeks of life may explain the difference. Studies designed to examine the hypothesis are in progress.

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