THE EFFECT OF THE ORAL ADMINISTRATION OF 987P MONOCLONAL ANTIBODY ON GNOTOBIOTIC PIGS CHALLENGED WITH 987P-POSITIVE *ESCHERICHIA COLI*

By

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ABSTRACT

THE EFFECT OF THE ORAL ADMINISTRATION OF 987P MONOCLONAL ANTIBODY ON GNOTOBIOTIC PIGS CHALLENGED WITH 987P-POSITIVE *ESCHERICHIA COLI*

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This study determined the effect of 987P monoclonal antibody (MCA) on the *in vitro* adhesion of 987P-positive *Escherichia coli* (*E. coli*) to pig intestinal brush borders and the effect of direct passive oral immunization with 987P MCA on clinical signs, lesions, *in vivo* adhesion and enterotoxin production in gnotobiotic pigs challenged with 987P-positive *E. coli*.

Using a brush border adhesion test, ileal samples from 29 pigs were used to determine if *in vitro* adhesion of 987P-positive *E. coli* to brush borders could be prevented by 987P MCA. Brush borders and bacteria were mixed with several concentrations of 987P MCA, titer of 10,000, for a total of 8 treatments per sample. Adhesion was present in all 29 samples tested in the absence of MCA. A statistically significant inhibition of adhesion of 987P-positive *E. coli* to brush border fragments by 987P MCA was present with all treatments.

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Neonatal gnotobiotic pigs from 8 litters were given 0.5 to 12 ml of 987P MCA, challenged with 987P-positive _E. coli_ 0.5 to 2 hours later and observed clinically for signs of enteric colibacillosis. Clinical signs, bacterial counts, and gross, histologic and scanning electron microscopic lesions were similar in control and treated animals. All, except 2 animals, had diarrhea, and gross lesions were minimal. Adhesion and colonization based on microbiologic, histologic and scanning electron microscopic examinations were limited in both control and treated animals, making it difficult to assess the efficacy of the MCA.

Enterotoxin production was determined in 2 of the 8 litters of gnotobiotic pigs. Pig ileal mucosa cyclic guanosine monophosphate (cGMP) content was determined using a cGMP radioimmunoassay. No statistically significant difference in cGMP values was present between treated and control animals. Using a suckling mouse assay, all intestinal content filtrates tested were negative for heat-stable enterotoxin. At the time of euthanasia, substantial amounts of enterotoxin were not present in the pigs.

In vitro, the 987P MCA was effective in the inhibition of adhesion of 987P-positive _E. coli_ to brush borders. However, the 987P MCA was ineffective in the

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prevention of diarrhea in gnotobiotic pigs challenged with 987P-positive *E. coli*.

DEDICATION

To the three Maes in my life,

Julia Mae Grimes, Willie Mae Moss and Eula Mae Grimes

and

my father,

George Washington Grimes

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ADP	adenosine 5'diphosphate
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine monophosphate
ELISA	enzyme linked immunosorbent assay
ETEC	enterotoxigenic *Escherichia coli*
GalNac	N-acetylgalactosamine
GluNac	N-acetylglucosamine
GDP	guanosine 5'-diphosphate
G_s	guanyl nucleotide binding regulatory protein
GTP	guanosine 5'-triphosphate
Ig	immunoglobulin
KDa	kilodalton
LT	heat-labile enterotoxin
LT_h	human heat-labile enterotoxin
LT_p	porcine heat-labile enterotoxin
MCA	monoclonal antibody
MRHA	mannose-resistant hemagglutinating activity
MSHA	mannose-sensitive hemagglutinating activity
nm	nanometers
pI	isoelectric point
RIA	radioimmunoassay
ST	heat-stable enterotoxin

INTRODUCTION

Worldwide, enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea in neonatal pigs, calves and lambs, as well as in children. Two virulence determinants are necessary for *Escherichia coli* (*E. coli*) to cause diarrhea: 1) enterotoxin production and 2) pilus production. Four antigenically distinct pili, K88, K99, 987P and F41, are present on separate strains of *E. coli* capable of infecting pigs (Ørskov *et al.*, 1961; Moon *et al.*, 1977; Nagy *et al.*, 1976; Morris *et al.*, 1983). Pili allow *E. coli* to attach to specific receptors on the mucosal surface of the pig's small intestine. Once attached *E. coli* produce enterotoxins which cause hypersecretion, diarrhea, dehydration and death (Moon *et al.*, 1979).

Since adhesion of ETEC to the intestine is an essential prerequisite for the development of enteric colibacillosis, antibody-mediated prevention of bacterial adhesion is a potential defense mechanism against *E. coli*. In neonatal animals, such antibody may be acquired from colostrum and milk. Vaccination of sows prior to farrowing helps to induce protective levels of antibodies in the sow's colostrum and milk, thus providing passive immunity to the newborn pig. Currently, several vaccines have been developed for the protection of pigs against neonatal enteric colibacillosis. Research has demonstrated that protection correlates with anti-pilus antibodies in the colostrum and milk (Acres *et al.*, 1979; Nagy *et al.*, 1978; Rutter *et al.*, 1976). Several researchers believe that colostral antibodies prevent the colonization of ETEC by blocking adhesion (Nagy *et al.*, 1978; Rutter, 1975; Rutter *et al.*, 1973, 1976).

Recently, an alternative method for direct passive immunization of newborn animals against ETEC, the oral administration of *E. coli* pilus-specific monoclonal antibody (MCA), has been developed. However, knowledge concerning the effectiveness of pilus-specific MCAs is limited.

One of the first reports involved the oral administration of *E. coli* K99 MCA to calves (Sherman *et al.*, 1983). In that study, there was a statistically significant reduction in the mortality rate and the severity and duration of diarrhea in the treated versus the control calves. However, there was no difference in the incidence of diarrhea. Explanations postulated for the diarrhea were: 1) the amount of MCA used may not have completely blocked colonization and 2) the number of bacteria in the challenge inoculum might have been large enough to produce sufficient enterotoxin, without adhesion to the mucosa, to cause diarrhea.

Studies with pigs using K88- (Foged *et al.*, 1986; Sadowski, 1984), K99- (Mainil *et al.*, 1987; Sadowski *et al.*, 1983) and 987P MCAs have also been performed. Few animals were used in the studies which were primarily clinical in nature. The effectiveness of the antibody varied from protection against challenge, based on an increased survival rate and a delayed onset of diarrhea, to no prophylactic effect.

In the present study, 987P MCAs were given to gnotobiotic pigs in an attempt to protect the pigs from diarrhea due to 987P-positive *E. coli.* The objectives of this study were:

1. To determine if the *in vitro* adhesion of 987P-positive *E. coli* to pig intestinal brush borders could be prevented by the administration of *E. coli* 987P MCA.
2. To determine if the clinical signs and lesions produced by 987P-positive *E. coli* in gnotobiotic pigs could be prevented by the oral administration of *E. coli* 987P MCA.
3. To determine if the *in vivo* adhesion of 987P-positive *E. coli* to the intestinal mucosa of gnotobiotic pigs could be prevented by the oral administration of *E. coli* 987P MCA.

4. To determine if enterotoxin production in gnotobiotic pigs challenged with 987P-positive *E. coli* could be affected by oral MCA administration.

LITERATURE REVIEW

Escherichia coli, a gram negative, motile or nonmotile, nonspore-forming rod, belonging to the family *Enterobacteriaceae*, is a normal inhabitant of the lower intestinal tract of all warm-blooded animals (Bruner and Gillespie, 1973). However, when pathogenic strains of *E. coli* are present in the small intestine in large numbers, diarrhea occurs.

Since 1899, *E. coli* has been thought to be a cause of diarrhea in neonatal pigs (Jensen, 1948). Several different types of *E. coli* have been reported to cause diarrhea in domestic animals and man. *Escherichia coli* associated with diarrhea are divided into 5 categories: 1) enterotoxigenic, 2) enteropathogenic, 3) enteroinvasive, 4) enterohemorrhagic and 5) enteroadherent. Classifications are based on the bacterium's virulence properties, interactions with the intestinal mucosa, clinical syndromes, epidemiology and O:H serotypes (Levine, 1987).

A vast amount of literature has been published regarding ETEC infections. There are several current general reviews about ETEC in the literature (Gaastra and de Graaf, 1982; Gross and Rowe, 1985; Klemm, 1985) The emphasis of this literature review will be placed on ETEC infections in swine and details relevant to the present research.

Clinical Signs

Piglets from 12 hours of age to several weeks of age, with a peak incidence occurring at 3 days of age, are susceptible to enteric colibacillosis. Usually several or all the pigs in a litter are affected. Pigs farrowed by first litter gilts have a greater incidence of enteric colibacillosis than do pigs farrowed by sows (Blood et al., 1983; Wilson, 1986).

Infected piglets have yellowish or brownish feces which vary from a pasty to a watery consistency. The anal or perineal region may be inflamed from contact with the alkaline feces. The animals have rough hair coats and normal to subnormal temperatures. They are also anorectic, depressed and weak. As the condition progressively worsens, the animals become dehydrated and emaciated. Pigs commonly die within 24 hours after the onset of clinical signs (Blood et al., 1983; Wilson, 1986).

Lesions

Gross lesions are minimal. The intestines of the affected pigs may be normal, hyperemic, distended with fluid or gas, or flaccid. Clotted milk may be present in dilated stomachs (Sojka, 1965). Venous infarcts may be present on the greater curvature of the stomach. Catarrhal enteritis may also be present (Blood *et al.*, 1983; Jubb and Kennedy, 1985; Wilson, 1986).

Histologically, few lesions are present in the intestines. Smith and Jones (1963) reported that no inflammatory changes were present in the intestinal tracts of pigs. Christie and Waxler (1972) reported that a mild neutrophilic infiltration of the intestinal villi, hydropic degeneration in villous epithelial cells and edema were present in gnotobiotic pigs infected with *E. coli*. Bacilli may be seen attached along the margins of the small intestinal villi (Wilson, 1986). Congested blood vessels may be present in the lamina propria, and villous atrophy may be present in localized areas of the small intestine (Wilson, 1986).

With both transmission and scanning electron microscopy, bacteria are seen attached on their sides and poles to small intestinal epithelial cells (Hohman and Wilson, 1975). Villous atrophy may also be seen, with the loss of degenerative epithelial cells occurring primarily at the villous extrusion zones. Moon (1982) suggested that the loss of villous epithelial cells indicated an accelerated epithelial replacement rate.

Pathogenesis

Following oral ingestion of *E. coli*, 2 important factors are required for the organism to cause diarrhea: 1) pilus production and 2) enterotoxin production.

Pili

Pili, a Latin term for hairs or hair-like structures, are filamentous organelles projecting from the bacterial surface. Brinton first used the term in association with these structures in 1959. Prior to that time, Duguid *et al.* (1955) had used the term fimbriae, meaning fringe, threads or fibers in Latin. Both terms are presently being used in the literature.

Two important functions are associated with pili: 1) they increase the active surface area which may facilitate membrane associated activities such as respiration and nutrient uptake and 2) they act as attachment organelles (Ottow, 1975). The latter function plays an important role in the pathogenesis of neonatal enteric colibacillosis.

Structurally, the pili present on ETEC are composed of numerous repeating protein subunits (Isaacson, 1977; Mooi and de Graaf, 1979). A single pilus consists of from several hundred to a thousand identical subunits

(Korhonen et al., 1985). The subunits are held together by noncovalent forces; no intersubunit cysteine bridges have been discovered (Klemm, 1985). The lack of sulfur-containing amino acids suggests that no disulfide bonds are present in the pili. Pili acting as adhesive organelles are peritrichously arranged on the bacterial surface (Ottow, 1975). The pili on ETEC were originally thought to be capsular (K) antigens and were designated K88 and K99. However, K antigens are polysaccharide in nature, and pili are composed almost entirely of protein. Hence their designation as K antigens is not correct.

Following entry of bacteria into the small intestine, there are several non-immunological defense mechanisms, such as gut peristalsis, villous pumping, and flowing ingesta and mucus, which tend to rapidly wash bacteria out of the small intestine (Dixon, 1960; Moon, 1980). Pili, however, enable ETEC to overcome these natural defense mechanisms by allowing bacteria to attach and rapidly proliferate in the small intestine to numbers comparable to those normally found in the sluggishly motile, avillous large intestine (Bertschinger et al., 1972; Jones and Rutter, 1972).

Evidence supporting the fact that pili on ETEC facilitate adhesion has been demonstrated in vivo and in vitro. Several early reports demonstrated the close microscopic association of ETEC with small intestinal villi (Arbuckle, 1970, 1971; Drees and Waxler, 1970; Moon *et al.*, 1971; Staley *et al.*, 1969). Bertschinger *et al.* (1972), showed that enteropathogenic *E. coli* (EEC) are more likely to be associated with the intestinal epithelium than nonenteropathogenic *E. coli* (NEEC). The EEC were present adjacent to the brush border along the entire villus, but the NEEC were randomly distributed in the central lumen.

In conventionally reared pigs, Jones and Rutter (1972) demonstrated that the K88-positive *E. coli* was able to adhere to the mucosa of the small intestine and cause diarrhea, compared to the K88-negative mutant which was unattached, was distributed throughout the lumen and did not cause diarrhea. These results indicated that the K88 pilus is responsible for adhesion of K88-positive bacteria to the small intestinal mucosa and that adhesion is required for the virulence of K88-positive bacteria in pigs. Supportive evidence for pili facilitating adhesion was also provided by Nagy *et al.* (1976, 1977) when strain 987P, a K88-negative piliated strain of ETEC, was also shown to adhere to the small intestine.

The *in vitro* adhesion of piliated ETEC has been demonstrated with the brush border technique (Sellwood *et al.*, 1975), intestinal epithelial cell technique (Isaacson *et al.*, 1978; Nagy *et al.*, 1977; Wilson and Hohman, 1974), the intestinal villus technique (Girardeau, 1980) and hemagglutination studies (Evans _et al._, 1979).

The pili most commonly present on strains of _E. coli_ capable of causing diarrhea in neonatal pigs are K88, K99 and 987P. The F41 pilus maybe present on piglet strains of ETEC (Morris _et al._, 1983). A pilus distinct from the former pili, with characteristics of mannose-resistant hemagglutinating activity (MRHA) to porcine erythrocytes, has also been described (Aning and Thomlinson, 1983). Recently, another adhesive factor, designated F42, with MRHA to human, sheep, guinea pig and chicken erythrocytes has been isolated from pigs (Yano _et al._, 1986). It has also been suggested that the common or type 1 pili may also be important in the pathogenesis of enteric colibacillosis (Jayappa _et al._, 1983, 1985).

Strains of ETEC may produce more than 1 type of pilus antigen. Morris _et al._ (1980a) described a bipiliated strain producing both the K99 and F41 pilus antigens. Also, ETEC strains have been shown to produce both K88 and 987P pili (Schneider and To, 1982; Suarez _et al._, 1987).

Pili are also present on calf, lamb and human strains of _E. coli_. The pili present on calf strains of ETEC are K99 (Ørskov _et al._, 1975), F41 (Morris _et al._, 1980a; To, 1984b), F(Y) (Morris _et al._, 1985; Pohl _et al._, 1982; Shimizu _et al._, 1987) and 31A (Contrepois _et al._, 1986; Shimizu _et al._, 1987). Lamb ETEC strains possess the K99 pilus (Ørskov et al., 1975). Human ETEC strains possess colonization factor adhesin (CFA)/I (Evans et al., 1975), CFA/II (Evans and Evans, 1978) and putative colonization factor (PCF) 8775 (Thomas et al., 1982). In an attempt to standardize pilus terminology, Ørskov and Ørskov (1983) have suggested a new nomenclature system. In their system, the previously mentioned pili are named the following: F1 (type 1), F2 (CFA/I), F3 (CFA/II), F4 (K88), F5 (K99), and F6 (987P). The new terminology has not been universally accepted. Only those pili present on pig strains of ETEC will be discussed.

The K88 Pilus. Of the 3 most commonly found pili, the K88 pilus was the first to be discovered and consequently has had the most written about it. Ørskov et al. first described the K88 pilus in 1961. The extrachromosomally transferred K88 pilus is encoded for by a 50 megadalton plasmid (Ørskov et al., 1966; Shipley et al., 1978). Chemically, the K88 pilus has been identified as a protein, containing all the common amino acids except cysteine, with no carbohydrate present (Anderson et al., 1980; Mooi and de Graaf, 1979; Stirm et al., 1967b). Structurally, Stirm et al. (1967a) described it as a flexible, filamentous antigen with a diameter of 7-11 nanometers (nm) and a length of 100 to 150 nm. Wadström et al. (1979) estimated the K88 pilus's diameter to be 2.1 nm. The K88 pilus is a homopolymer of approximately 100 hydrophobically linked subunits whose monomeric molecular weight ranges from approximately 23,000 to 27,000, depending upon the serologic variant (Anderson et al., 1980; Klemm, 1981; Mooi and de Graaf, 1979).

Serologically, there are 3 variants of the K88 antigen, K88ab, K88ac and K88ad (Guinee and Jansen, 1979a); Ørskov et al., 1964). The isoelectric point (pI) of the 3 K88 variants pooled together is 4.2 (Mooi and de Graaf, 1979). The common antigenic determinant, a, combines with the variable antigenic factor designated b, c, or d. The variants are antigenically different due to amino acid sequence differences in the pilus proteins. Amino acid sequences corresponding to the antigenic variables a, b, c, and d have been predicted (Gaastra and de Graaf, 1982; Gaastra et al., 1979, 1981, 1983; Klemm, 1981; Klemm and Mikkelsen, 1982). Regardless of the antigenic variant, the K88 subunit consists of 264 amino acid residues.

The existence of the K88ab and K88ac variants has been known since 1964, but the K88ad variant was discovered recently. The K88ab variant was common in the 1960s, but its presence is rare today. Now, most K88-positive E. coli isolated from infected pigs possess either the K88ac of K88ad variant (Gaastra and de Graaf, 1982; Guinee and Jansen, 1979b). A recent survey suggests that the K88ac variant is the predominant variant in the major pork-producing region of the United States based on a study examining 415 K88-positive strains of _E. coli_ present in 9 states from 1976 to 1985 (Westerman _et al._, 1988). In fact, all 415 strains of _E. coli_ were found to possess the K88ac pilus. No other serologic variants were found.

It is unclear whether the amino acid substitutions present in the K88 pilus variants indicate an attempt of the bacteria to evade the host's immune system or whether they indicate bacterial adaptation to altered receptor sites on pig small intestinal brush borders. It has been suggested that the K88ac variant was derived from the K88ab variant and that the K88ab variant has undergone selective pressure by large increases in the amount of antibody directed against it on pig farms (Guinee and Jansen, 1979a). The very low number of base substitutions not resulting in amino acid substitutions, only 5 out of 47, suggests that the K88ab and K88ad variants have not evolved from each other in a normal way but that they must have been subject to intensive selective pressure, such as might result from an intensive vaccination program (Gaastra _et al._, 1983). The other possibility is that the number of resistant pigs (pigs without receptors for the K88 pilus) may have caused a selection toward _E. coli_ with an alteration in their pili to match receptor sites already present on the pigs' small intestinal epithelium (Gaastra and de Graaf, 1982).

Molecular analysis of pilus determinants has shown that they encode multiple gene systems with the interaction of several genes being required for the assembly of the pili (Dougan *et al.*, 1983; Kehoe *et al.*, 1981; Mooi *et al.*, 1979). Six structural genes are involved in the production of the K88 pilus. Mooi *et al.* (1979) isolated the genetic determinant for K88ab by molecular cloning. The resultant recombinant plasmid appeared to code for 5 polypeptides, with molecular weights of 17 kilodaltons (KDa), 26 Kda, 27 KDa, 27.5 KDa and 81 KDa, which were precursors in the biosynthesis of K88ab (Mooi *et al.*, 1981, 1982). The 17, 27 and 27.5 KDa polypeptides are located in the periplasmic space, the 81 KDa polypeptide in the outer membrane and the 26 KDa polypeptide (the K88ab pilus subunit) is located on the cell surface, probably attached to the outer membrane (van Doorn *et al.*, 1982). The 81 KDa probably serves to anchor the pilus subunits to the outer membrane (Mooi *et al.*, 1983). The sixth polypeptide, A, a pilus-like protein, appears to be necessary for the export of the K88ab subunits, and it has also been suggested that polypeptide A and the K88ab pilus are part of the same structure, with the K88ab pilus subunit probably representing the major component. Polypeptide A might serve as a link between the K88ab pilus and the anchorage protein. Alternatively, the 2 polypeptides might form separate structures on the cell surface, the synthesis being interdependent. Thus, the colonization of some surfaces by K88-positive *E. coli* might require at least 2 extracellular proteins (Mooi *et al.*, 1984).

Escherichia coli bearing the K88 pilus show MRHA for guinea pig (Jones and Rutter, 1974; Stirm *et al.*, 1967) and chicken erythrocytes. The K88 pilus is species specific. Strains of *E. coli* that are K88-positive are able to colonize pig intestine *in vivo* and cause diarrhea, but K88-positive ETEC have not been reported as enterotoxigenic in other species (Moon *et al.*,1977; Smith and Halls, 1967). Deneke *et al.* (1984) showed that ETEC with K88ac or K88ab pili bind to isolated human small intestinal epithelial cells. Also, Tzipori *et al.* (1984) have determined that K88 ab- and K88ac-positive *E. coli* attach *in vitro* to epithelial cells from both foals and adult horses. *Escherichia coli* bearing K88 pili are found in pigs up to several weeks of age.

The K99 Pilus. Ørskov *et al* first described the Kco antigen present on calf and lamb enterotoxigenic strains of *E. coli* in 1975. In 1977, the K99 pilus was described on pig strains of ETEC (Moon *et al.*, 1977). The K99 pilus is encoded for by a transmissible plasmid (Smith and Linggood, 1972) with a molecular weight of 57 megadaltons

(So et al., 1976). Isaacson (1977) concluded that the K99 antigen was a pilus based on its subunit structure, external surface location and rod-like shape. It has been described as a thin, flexible filament with an open structure and stretched subunits (Jones and Isaacson, 1983). The pilus's length was reported to be 130 to 160 nm, and its diameter was reported to be approximately 8 nm (Altman et al., 1982; Isaacson, 1977). Isaacson et al. (1981) have since reported the pilus's diameter to be 4.8 nm.

The K99 pilus is protein in nature and lacks the sulfur-containing amino acids, cysteine and methionine (Altman et al., 1982). In the past, there has been a great deal of controversy concerning the molecular weight and characterization of the K99 pilus with disparate reports based on different isolation procedures originating from several laboratories (Altman et al., 1982; Chantner, 1982; de Graaf et al., 1980; Isaacson, 1977, 1978; Morris et al., 1977, 1978a,b). Morris et al., (1980a) have reported that there are 2 pilus subunits present in serogroups 09 and 0101 with different molecular weights and ionic charges. The cationic and lower molecular weight component was the K99 pilus, and the high molecular weight, anionic component was the F41 pilus which has been shown to be a pilus with adhesive abilities (Morris et al., 1982). The molecular weight of the K99 pilus subunit was reported by de Graaf (1980) to be 18,500 and by Chanter (1982) to be 19,000.

Horse and sheep erythrocytes show MRHA with K99-positive _E. coli_ (Burrows _et al._, 1976, Gaastra and de Graaf, 1982; Isaacson, 1978; Morris _et al._, 1980a). With a pI of 9.5, K99 is the only pilus antigen with a basic pI (de Graaf _et al._, 1980). The nucleotide (Roosendaal _et al._, 1984) and amino acid sequences (de Graaf _et al._, 1980 and de Graaf and Roorda, 1982) of the K99 pilus have been determined. Structural genes encoding 7 polypeptides involved in K99 biosynthesis have also been described (de Graaf _et al._, 1984).

The K99 pilus is not species specific. Calf, lamb and pig strains of _E. coli_ bearing K99 pili are pathogenic (Moon _et al._, 1977; Ørskov _et al._, 1975; Smith and Linggood, 1972). The K99-positive _E. coli_ are found in pigs less than 2-weeks-old (Moon _et al._, 1980).

The 987P Pilus. The 987P pilus was first determined to facilitate intestinal adhesion and colonization in 1976 (Nagy _et al._, 1976). Composed primarily of protein, the 987P pilus has also been shown to possess an amino sugar component (Fusco _et al._, 1978; Isaacson and Richter, 1981). The 987P pili are morphologically indistinguishable from type 1 pili. The 2 types of pili have a tight, rigid structure and are composed of fewer subunits per turn than K88 and K99 pili, creating an axial hole as determined by electron microscopy (Jones and Isaacson, 1983). Strong hydrogen bonds appear to be present between the 987P subunits (Schifferli, 1987). The pili have diameters of 7 nm and obtain lengths up to 1 μm (Fusco et al., 1978; Isaacson and Richter, 1981).

The molecular weight of the 987P pilus's subunit was first reported as 18.9 KDa by Fusco et al., (1978) and has since then been reported to be 20 KDa by Isaacson and Richter (1981). The 987P pilus is not plasmid determined (Isaacson et al., 1977; Nagy et al., 1977) but is believed to be genetically encoded for by a chromosome (Gaastra and de Graaf, 1982). The pilus has a pI of 3.7 (Isaacson and Richter, 1981). Mannose-resistant hemagglutination of horse, guinea pig, sheep, rabbit, pig and cow erythrocytes does not occur with 987P-positive E. coli (Isaacson et al., 1977; Isaacson and Richter, 1981), but weak MRHA of chicken erythrocytes has been demonstrated by Awad-Masalmeh et al. (1982).

In a study by Moon et al. (1980) to determine the prevalence of pili in ETEC isolated from neonatal pigs with diarrhea, 76% of the E. coli strains were able to produce 987P pili. In a later study by Brinton et al. (1983), 987P pili were also reported to be the most common pili, excluding type 1 pili, in the United States. The more common occurrence of the 987P pilus compared to the other type of pili is probably due to the chromosomal locus of the pilus's gene (Brinton *et al.*, 1983).

The 987P pilus is considered to be species specific as a virulence factor in pigs, even though it has been found on calf strains of *E. coli*. In calves, 987P piliated strains of *E. coli* appear to be harmless (Moon, 1978b). The 987P pilus is found on strains of ETEC present in pigs less than 2 weeks of age.

The F41 Pilus. The F41 pilus was first described by Morris *et al.* in 1980 and referred to as F41 in 1982. The pilus was first found in association with K99-producing ETEC of serogroups 09 and 0101 (Morris *et al.*, 1980a, 1983). The F41 pilus appears to be the same pilus that was described by Moon *et al.* (1980) and Awad-Masalmeh *et al.* (1982). The F41 pilus is thin and flexible (Moon *et al.*, 1980) and has a diameter of 3.2 nm, a pI of 4.6 and a molecular weight of 29.5 KDa (de Graaf and Roorda, 1982). It has the largest fimbrial subunit that has been found to date.

Escherichia coli possessing the F41 pilus hemagglutinate guinea pig and sheep erythrocytes strongly and horse erythrocytes to a lesser degree (Gaastra and de Graaf, 1982; Morris *et al.*, 1980a). However, de Graaf and Roorda (1982) indicated that the F41 pilus caused strong MRHA of human and guinea pig erythrocytes and weak MRHA of sheep and horse erythrocytes. To (1984b) reported that the F41 pilus agglutinated chicken, goat and pig erythrocytes. The amino acid sequence of the F41 pilus has been determined (de Graaf and Roorda, 1982). The F41 antigen is encoded for by a chromosome, and the genes required for F41 production have been cloned (Moseley *et al.*, 1986).

Morris *et al.* (1983) were the first to discover the F41 pilus on piglet strains of ETEC. Strains of *E. coli* possessing F41 alone have been shown to be virulent in pigs (Awad-Masalmeh, 1982; Morris *et al.*, 1982, 1983; To, 1984b) and calves (To, 1984b). *Escherichia coli* bearing the F41 pilus also colonize the intestinal epithelium of lambs (Morris *et al.*, 1980b).

Type 1 Pili. The type 1 pili are generally not believed to be involved in the pathogenesis of enteric colibacillosis. However, some authors have suggested that type 1 pili may indeed play a role in the colonization of the small intestine by ETEC.

The previously-described pili present on ETEC are distinctly different from the common or type 1 pili of *E. coli*. A major distinction is that ETEC pili have MRHA, compared to the mannose-sensitive hemagglutinating activity (MSHA) of type 1 pili (Duguid *et al.*, 1955; Evans *et al.*, 1979). Type 1 pili of enterobacteria, including *E. coli*, show the same pattern of hemagglutination specificity for erythrocytes of different animal species.

They show strong MSHA for guinea pig, chicken, horse and monkey erythrocytes, moderate MSHA for human erythrocytes, weak MSHA for sheep erythrocytes and no MSHA for cow erythrocytes (Duguid et al., 1955, 1979; Duguid and Old, 1980). Type 1 pili measure 7-8 nm in width and 0.2 to 2 μm in length (Brinton, 1959, 1967, 1978). The pili are chromosomally encoded (Brinton, 1959, 1965, 1967).

It has been determined that the type 1 pilus family consists of at least 12 different types of pili (Brinton et al., 1983). However, all serotyped type 1 pili isolated from neonatal swine with diarrhea have been serotype 2, type 1 pili. Some experiments suggest that K88-positive E. coli may require serotype 2, type 1 pili for virulence. Also, a porcine isolate of E. coli phase cloned to express only serotype 2, type 1 pili caused experimental colibacillosis in a significant portion of pigs, indicating that serotype 2, type 1 pili may act as independent pilus virulence factors for swine neonatal enteric colibacillosis (Jayappa et al., 1983). Jayappa et al. (1983, 1985) have reported that ETEC with type 1 pili adhere to and colonize the small intestine of pigs and that this adhesion could be prevented by type 1 antiserum. Immunofluorescence demonstrated the presence of E. coli expressing type 1 pili on the brush borders of colostrum deprived newborn pigs, thus suggesting the involvement of the pili in colonization. Anti-type 1 serum was given to newborns prior to challenge, and the number of gut associated _E. coli_ compared to controls was decreased. In another study, a strain of _E. coli_ possessing type 1 pili, but no other known swine pili, with MSHA to guinea pig erythrocytes caused diarrhea in hysterectomy derived colostrum-deprived pigs (Nakazawa _et al_, 1986). Type 1 pili have also been shown to adhere _in vitro_ to porcine intestinal epithelial cells (Isaacson _et al._, 1978). A type 1 pilus vaccine has been shown to be protective in humans challenged with the same serotype expressed in the vaccine (Levine _et al._, 1982). Reports have indicated that a vaccine containing type 1, serotype 2 pili, as well as K88, K99 and 987P pili, is much more effective in the prevention of swine enteric colibacillosis than similar vaccines which lack the type 1 pili (Brinton _et al._, 1983; Jayappa _et al._, 1983). However, in a study by To _et al._ (1984) it was reported that not only were type 1 pili not protective antigens for ETEC infections, but also that the challenge strain didn't produce type 1 pili in the small intestine of the pigs during colonization.

Receptors

Pili facilitate attachment of bacteria to specific receptors present on the small intestinal mucosa (Faris _et al._, 1980; Evans and Evans, 1978; Smit _et al._, 1984). Ultrastructural observations indicate that strong mucosal attachment of ETEC, sufficient to overcome host peristaltic clearing mechanisms, can be achieved by the formation of a large number of bonds between pili and host mucosal receptors (Knutton _et al._, 1984). Less specific hydrophobic and ion electrostatic interactions between the bacterial glycocalyx and intestinal epithelium are also involved in the attachment of bacteria to the small intestine (Wadström _et al._, 1979).

Present knowledge about the actual nature of the receptors to which K88, K99, 987P and F41 attach is limited. Research is currently ongoing to determine the chemical composition of the receptors. Reports indicate that chemically the receptors are composed of glycolipids or glycoproteins (Gibbons _et al._, 1975; Kearns and Gibbons, 1979). Depending upon the assay involved, various sugars, glycoproteins or glycolipids may inhibit the binding of piliated _E. coli_ to brush borders, epithelial cells or erythrocytes.

Gibbons _et al._ (1975) determined that glycoproteins with a terminal β-D-galactosyl structure were able to inhibit the hemagglutination of guinea pig erythrocytes by K88 pili, suggesting that the glycoprotein might bind with the K88 pilus and inhibit hemagglutination due to its resemblance to the K88 receptor. The β-galactosyl residues have also been shown to play an important role in the binding of K88 pili to brush borders (Sellwood, 1980b). Binding of K88 pili to brush borders occurs from

4°C to 37°C, while binding to the guinea pig erythrocytes only occurs at 4°C, indicating that the fit for the natural receptors is better than that of the guinea pig erythrocyte receptors (Sellwood, 1980b; Gibbons *et al.*, 1975).

Glycoproteins with N-acetylglucosamine (GluNac) and N-acetylgalactosamine (GalNac) as terminal sugars inhibit binding of the K88 antigen to brush borders and, to a lesser degree, sugars alone, such as GluNac, GalNac and N-acetylmannosamine, can also inhibit binding (Anderson *et al.*, 1980). The sugar D-galactosamine has also been reported to inhibit binding of the K88 pilus to brush borders (Sellwood, 1984a,b).

Glycolipids have also been shown to have K88 receptor activity (Sellwood and Kearns, 1979; Kearns and Gibbons, 1979). The brush border receptor for the K88 pilus reacts chemically in a way similar to that of the cholera toxin receptor which is a G_{M1} ganglioside. However, according to Sellwood and Kearns (1979), it doesn't really appear to be a ganglioside.

Hemagglutination tests have been performed on erythrocytes from several species in an attempt to identify specific receptor sites. Parry and Porter (1978) demonstrated that K88ab adhered strongly to chicken erythrocytes but not to the extent of that seen on the brush borders of intestinal epithelial cells. Guinea pig erythrocytes were agglutinated by both K88ab and K88ac pilus preparations and live organisms. They concluded that both the a and the b or c determinants of the K88 pilus for K88ab and K88ac are involved in adhesion to intestinal epithelial cells and guinea pig erythrocytes. However, in the chicken erythrocytes, adhesion of the pilus appears to involve only the K88b determinant's receptor on the erythrocyte (Parry and Porter, 1978). The hemagglutination reaction is easier to study, but the adhesion is not as specific as that seen with the natural receptors on epithelial cells.

There may not be a separate brush border receptor for each of the serologic variants of K88-positive E. coli. Bijlsma et al. (1982) showed that blocking the receptor site with 1 serologic variant of the K88 antigen also inhibited the adhesion of other K88 variants. For example, when brush borders from pigs susceptible to all 3 serologic variants of E. coli were exposed to the K88ab antigen and the receptor sites were blocked, K88ad- and K88ac-positive strains of E. coli were no longer able to adhere. However, adhesion of K88ad to brush borders from 1 pig susceptible to all 3 serologic variants of K88 was not inhibited by K88ab or K88ac antigen. This suggests that the receptor site for K88ad in brush border from pigs susceptible to all 3 serologic variants may have 2 configurations which can only be detected by receptor blocking. Bijlsma *et al.* (1982) consider that only 1 receptor, depending upon its modifications, will allow attachment of 1, 2 or all 3 of the K88 serologic variants.

Wilson and Hohmann (1974) believe that the K88a antigenic component doesn't affect the ability of bacteria to attach to intestinal mucosal cells. They found that the antigen necessary for blocking adhesion was the c or b component. Bijlsma *et al.* (1982) say that it is unlikely that the antigenic components b, c, or d are exactly the same as the adhesion component of the K88 pilus because K88ac antigen is able to block K88ab and K88ad receptors of phenotype A brush border, yet K88c antibody doesn't react with K88b and K88d. Conversely, Parry and Porter (1978) were able to block the adhesion of K88ab- and K88ac-positive strains of *E. coli* to brush borders by using antisera specific for the K88a determinant. Antibodies against K88b and K88c antigens only inhibited homologous strains of K88-positive *E. coli*.

Intestinal epithelial cells are believed to have different receptors for different pili. Using strains of *E. coli* with K99 and 987P pili, binding of piliated *E. coli* to brush borders was inhibited by homologous pili, but not heterologous pili, which indicated that a difference in receptors existed (Isaacson *et al.*, 1978).

The K99 antigen has been shown to be specific for sialic acid (Faris *et al.*, 1980; Lindahl and Wadström, 1984; Smit _et al._, 1984). Substances with sialic acid as a major component were able to inhibit the binding of calf K99-positive ETEC to sheep erythrocytes using a MRHA test. The K99 surface hemagglutinin of ETEC recognize terminal GalNac and sialic acid residues of glycophorin and other complex glycoconjugates. The K99 pilus has also been shown to bind to glycophorin A on human erythrocytes (Lindahl _et al._, 1984) and to bind to NeuGc (2→3) lactosylceramide, a glycolipid, on equine erythrocytes (Smit _et al._, 1984). Lindahl and Wadström (1984) have suggested that the K99 erythrocyte receptor may be a glycophorin with a N-acetylneuramic acid 2-6 GalNac structure. Earlier, Morris _et al._ (1977) demonstrated with hemagglutination experiments that the K99 pilus was sensitive to the GalNac structure. Human, porcine and equine erythrocytes which are hemagglutinated by K99 pili all have similar glycophorin oligosaccharide structures (Lindahl and Wadström, 1984). Hemagglutination inhibition of K99-positive ETEC has also been inhibited with a glycoconjugate resembling the GM_2-ganglioside (Faris _et al._, 1980). The N-acetylneuramic acid portion appeared to play an important role in the inhibition of binding.

The F41 pilus may be specific for GalNac and have some affinity for GluNac (Lindahl and Wadström, 1984).

It appears the receptors for pili are capable of releasing _in vitro_ from brush borders or intestinal cell membranes. Kearns and Gibbons (1979) reported that plasma membranes prepared from positive brush borders lost 98% of their receptor activity. They were able to demonstrate that a supernatant fraction, obtained during the preparation of K88-positive brush borders, was able to enhance the adhesion of K88-positive E. coli to both positive and negative brush borders. Supernatant fractions obtained from negative brush borders did not enhance adhesion. Utilizing pig mucosal organ cultures, other researchers have also demonstrated the release of receptors for K88-positive E. coli into culture media (Staley and Wilson, 1983; Wilson et al., 1984).

Dean and Isaacson (1982) have identified a soluble 987P pilus receptor-containing fraction which was released from adult rabbit brush borders stored at 4°C. They have shown that the receptor is a low molecular weight, less than 14 KDa, acidic glycoprotein based on SDS gel electrophoresis (Dean and Isaacson, 1985b). The 987P receptor of adult rabbits was present along the entire small intestinal villous surface and in goblet cells, but in infant rabbits it was present only in goblet cells. The receptors in adult rabbits were spread equally throughout the jejunum and ileum. Material antigenically similar to the rabbit 987P receptor has been demonstrated in goblet cells in neonatal pig ileum. The identification of the 987P receptor in what appeared to be goblet cells was consistent with previous thought that the 987P receptor was a component of the glycocalyx. Support for the receptor being a component of glycocalyx is based on the acidic nature of the glycoprotein 987P receptor (Dean and Isaacson, 1985a), its release from brush borders upon storage (Dean and Isaacson, 1982) and the coadherence, or multilayered adherence, of 987P-positive bacteria to epithelial brush borders (Dean and Isaacson, 1982). It has been suggested that the 987P receptor is produced and secreted by goblet cells and that the 987P receptor is a lectin that must bind to brush borders to function as a receptor for the 987P pilus (Dean and Isaacson, 1985a). The 987P receptor is multivalent (Dean and Isaacson, 1985b). Charge, salting effects and perhaps a free amino group may be important factors in the 987P pilus-receptor interaction since the 987P pilus can be inhibited from binding to its receptor by high concentration of NaCl and by compounds containing a free amino group, such as NH_4Cl, amino sugar, lysine or ethanolamine, but not by neutral sugars or by glycine. Periodate oxidation of the 987P pilus also inhibits the pilus-receptor interaction which indicates that the pilus's carbohydrate may play a role in the interaction (Dean and Isaacson, 1985b).

Evidence also exists that receptors for K99 pili may also be released from brush borders *in vitro* upon storage at 4°C (Grimes *et al.*,1986).

The presence of receptors for K88 pili in pigs is genetically determined. Pigs lacking the receptor are resistant to diarrhea caused by K88-positive _E. coli_, whereas those with the receptor are susceptible (Sellwood _et al._, 1975). Moon _et al._ (1979) have stated that they have not encountered pigs congenitally resistant to colonization by _E. coli_ with K99 pili or 987P pili, indicating that all pigs have receptors for the K99 and 987P pili. In a study by Grimes _et al._ (1986) it was reported that most, if not all, pigs had intestinal receptors for K99 pili.

Receptors for the K88 pilus are located throughout the small intestine in pigs (Hohman and Wilson, 1975; Kohler _et al._, 1975; Moon _et al._, 1979; Smith and Linggood, 1971a). However, the association of K88-positive _E. coli_ with villi occurs most commonly in the ileum and least commonly in the duodenum (Arbuckle, 1976). Receptors for the K88 pilus are also present on mouse and calf epithelial cells (Runnels _et al._, 1980). _In vitro_, receptors for 987P (Dean and Isaacson, 1982; Isaacson _et al._, 1978; Nagy _et al._, 1977) and K99 (Isaacson _et al._, 1978) pili are present on pig jejunal and ileal brush borders. _In vivo_, in natural infections with 987P-positive _E. coli_, colonization appears to be confined to the ileum and large intestine (Isaacson _et al._, 1978; Moon _et al._, 1979), but ligated gut experiments have shown attachment to the jejunum also (Moon *et al.*, 1979; Nagy *et al.*, 1976). *In vivo*, K99-positive *E. coli* adhere to the ileum of pigs (Isaacson *et al.*, 1978; Moon *et al.*, 1977, 1979).

Mucus may play an important role in bacterial attachment to the small intestine (Forstner, 1978; Hoskins, 1978). Mucus contains mucin, which is composed of glycoproteins and glycolipids (Mantle *et al.*, 1981; Marshall and Allen, 1978; Schacter and Williams, 1982; Slomiany and Slomiany, 1984). As mentioned previously, some sugar structures of glycoproteins may serve as receptors for bacterial pili. Bacteria must pass through the mucus covering intestinal epithelial cells prior to their attachment to the cell, so glycoproteins present in mucus may act as false receptors for bacterial pili. This may prevent colonization by interfering with adhesion of the bacteria to the underlying epithelial cells. In one study, when mucus was removed, there was a significant increase in the number of *E. coli* adherent to ileal and colonic segments. Ileal mucin material, containing galactose and galactosamine, decreased the adhesion of *E. coli* to the ileal segments (Golderman *et al.*, 1985). Mouricout and Julien (1986, 1987) have suggested that in calves, diarrhea due to ETEC involves a bacterium-mucin recognition phenomenon in which bacterial pili and specific glycoprotein mucus receptors are important.

Sialic acid and galactose seemed to be partially responsible for the attachment of K99 pili, whereas F41 pili recognized desialylated receptors.

It has been suggested by Freter (1981) that to resist the flux of intestinal secretions and to colonize tissue, it may be sufficient for bacteria to bind to mucus alone. Even in cases where bovine ETEC bound to enterocytes, the _E. coli_ also interacted with the mucus (Freter, 1981; Laux _et al._, 1984; Mouricout _et al._, 1986). Thus, mucus may act as a site for replication and colonization before bacteria actually adhere to the small intestinal epithelial cells.

Laux _et al._ (1986) have identified 2 glycoprotein receptors, with molecular weights of 57 and 64 KDa, for the K88ab pilus in mouse small intestine. The receptors are present in both mucus and brush border membranes. A 91 KDa glycoprotein receptor present only in the brush border membranes was also identified. Adhesion of K88-positive _E. coli_ to the mouse small intestine was inhibited primarily by D-galactosamine. The presence of receptors for the K88ab pilus in mucus as well as on the brush border membrane may represent secreted mucus glycoproteins, and it has also been reported by Weiser (1984) that brush border membrane components are often released into the gut lumen (Laux _et al._, 1986). Receptors specific for the K88 pilus have been shown to exist in mouse, rat, rabbit and pig mucus (Laux et al., 1984; Slomiany and Slomiany, 1984).

Enterotoxins

Once attachment and colonization have occurred, ETEC are able produce enterotoxins. Enterotoxin production is the second important virulence determinant (Smith and Halls, 1967). Enterotoxins, when given by the intraluminal route, cause the net movement of fluid and electrolytes from plasma to the intestinal lumen (Dorner et al., 1976). Two types of enterotoxins, based on their thermolability, antigenicity and molecular weight, are produced by ETEC: 1) a heat-stable toxin (ST) and 2) a heat-labile toxin (LT). The heat-labile toxin is inactivated by heat at 60°C for 30 minutes, has a high molecular weight, and is antigenic (Gyles, 1974a; Gyles and Barnum, 1969). The heat-stable toxin is resistant to temperatures of 100°C for 30 minutes, has a low molecular weight, and is nonantigenic (Smith and Halls, 1967; Smith and Gyles, 1970). Both types of enterotoxin production are controlled by transmissible plasmids (Skerman et al., 1972; Smith and Halls, 1968).

Strains of ETEC may produce only 1 or both types of enterotoxin. Strains of ETEC that possess the K88 pilus may be associated with the production of LT, ST or both (Guinee and Jansen, 1979b; Gyles and Barnum, 1969; Smith and Gyles, 1970; Söderlind and Möllby, 1979). The 987P pilus is associated with the production of ST (Guinee and Jansen, 1979b; Moon et al., 1980). The K99 pilus is also associated with ST production (Guinee and Jansen, 1979b; Moon et al., 1976).

The Heat-Labile Enterotoxin. Although many different strains of ETEC produce LT, it is a highly homologous substance in all the strains. The LT toxin is divided into 2 types based on the origin of the isolate, human (LT_h) or porcine (LT_p) (Geary et al., 1982; Honda et al.; 1981c; Takeda et al., 1983; Tsuji et al., 1982; Yamamoto and Yokota, 1983). The 2 types of toxins have demonstrated minor antigenic and structural differences (Honda et al., 1981c; Tsuji et al., 1982), but the genes for the toxins are virtually identical (Dallas, 1983).

The LT was considered by Finkelstein et al. (1976) to be a heterogenous molecule whose molecular weight ranged from 35 KDa to 100 KDa, but Dorner et al. (1976) and Evans et al. (1976) reported a molecular weight of approximately 100 KDa. Finkelstein et al. (1976) suggested that the heterogeneity was due to proteolytic splitting of the molecule during purification. The toxin has now been shown to have a molecular weight of 86.5 KDa. Purification of the toxin may also have been complicated because LT is a cell-associated toxin, located in the periplasmic space (Wensink et al., 1978).

The LT toxin is similar, antigenically, structurally and functionally, to the cholera toxin of _Vibrio cholera_ (Clements and Finkelstein, 1978a,b; Gill _et_ 1981; Gyles, 1974a,b; Gyles and Barnum, 1969; Lindholm _et al._, 1983). Structurally LT, like cholera toxin, consists of 2 subunit fragments, 1 A subunit, with a molecular weight of 28 KDa, and 5 B subunits, with individual molecular weights of 11.5 KDa (Clements and Finkelstein, 1979). The B subunits are aggregated in a ring by tight noncovalent bonds. The A subunit is linked to and partially inserted in the B ring through weaker non-covalent interactions (Clements and Finkelstein, 1979; Dafni and Robbins, 1976; Gill _et al._, 1981; Kunkel and Robertson, 1979; Wolk _et al._, 1980). The LT B subunit has a DNA nucleotide sequence of 103 amino acid residues (Dallas and Falkow, 1980), and the A subunit consists of 236 amino acid residues (Spicer _et al._, 1981, Spicer and Noble, 1982).

The B subunit pentamer binds the LT toxin to a G_{M1} ganglioside (Donta and Viner, 1975; Moss _et al._, 1980; Svennerholm and Holmgren, 1978) in the mucosal cell membrane and creates a functional pore through which the A subunit enters the cell's cytosol (Holmgren, 1981). Also, in rabbit intestinal mucosal cells a glycoprotein, structurally related to the ganglioside, has been shown to act as an additional receptor for LT, but not cholera toxin (Holmgren _et al._, 1982; Holmgren, 1981). The action of the toxin has a lag time of greater than 30 minutes, but its effect is prolonged and unaffected by washing. The LT toxin is not tissue specific, it has a wide range of cells which it is capable of binding to and affecting (Guerrant _et al._, 1974; Kantor _et al._, 1974; Kwan _et al._, 1974; Mashiter _et al._, 1973; Zenser and Metzger, 1974).

The LT toxin, like cholera toxin, activates membrane bound adenyl cyclase which subsequently leads to an increase in intracellular cyclic adenosine 3',5'-monophosphate (cAMP) in epithelial cells of the small intestine (Evans _et al._, 1972; Hewlett, 1974). The activation of adenyl cyclase occurs after the movement of the A subunit of LT across the membrane and into the cytoplasm of the intestinal epithelial cell. A catalytically active fragment of the A subunit then splits intracellular nicotinamide adenine dinucleotide (NAD). If guanosine 5'-triphosphate (GTP) is present, the adenine 5'-diphosphate (ADP) ribose portion of NAD then covalently links onto the guanyl nucleotide binding regulatory protein (G_s) of the adenylate cyclase complex at the inner side of the basolateral membrane of the mucosal cell. Adenylate cyclase is then locked in an active form by the inhibition of an inherent feedback regulatory mechanism which normally involves the hydrolysis of GTP to guanosine 5'-diphosphate (GDP) and inorganic phosphorus, and this results in the net accumulation of intracellular cAMP (Gill et al., 1976,; Moss et al., 1979; Moss and Richardson, 1978). Choleragen, and possibly LT catalyzed ADP ribosylation of G_s appears to increase adenylate cyclase activity by 2 mechanisms. The toxin inhibits the GTPase associated with G_s, thus prolonging the life of the active GTP-G_s complex (Cassel and Selinger, 1977). Second, the ADP-ribosylation of G_s decreases it affinity for GDP, resulting in acceleration of GDP release and formation of the active GTP complex (Burns et al., 1982). Subsequently, there is a net secretion of an isotonic, alkaline fluid rich in electrolytes as a result of increased sodium, bicarbonate, and water secretion by crypt cells and decreased absorption of chloride coupled to sodium by the villus cells (Field, 1981; Moon, 1974; Moss and Vaughan, 1980). The increased secretion by the crypt cells leads to a secretory diarrhea (Moon et al., 1978a).

Field (1981) has suggested that calcium might be an important intracellular regulator of intestinal electrolyte transport, acting as the ultimate messenger for E. coli cAMP-induced secretion. It has been postulated that increased levels of cAMP cause a release of intracellular calcium which triggers activation of the calcium/calmodulin complex. This complex ultimately results in increased permeability of chloride from the intestinal crypt cells.

Several tests have been devised to test for the presence of LT - passive immune hemolysis (Evans and Evans, 1977), radioimmunoassay (RIA) (Greenberg et al., 1977), enzyme linked immunosorbent assay (ELISA) (Svennerholm and Holmgren, 1978; Yolken et al., 1977), Biken test (Honda et al., 1981a,b), latex particle agglutination (Finkelstein and Yang, 1983) and DNA hybridization (Moseley et al., 1980). Biological assays using animals (Moon and Whipp, 1971; Hamilton et al., 1978; Burgess et al., 1979) and tissue culture (Donta et al., 1974; Guerrant et al., 1974) have also been used to detect LT.

The Heat-Stable Enterotoxin. The second type of enterotoxin is ST. There is evidence that suggests that STs from different strains of ETEC represent a heterogenous group of E. coli (Guerrant et al., 1975). At least 2 distinct types of ST are known: 1) ST_I or ST_a which is methanol soluble, active in the infant mouse model and active in ligated jejunal segments of piglets 1 to 3 days old and 2) ST_{II} or ST_b which is methanol insoluble, inactive in the infant mouse model and active in ligated intestinal segments of 7-9-week-old weaned pigs (Burgess et al., 1978; Giannella, 1976; Kapitany et al., 1979a,b; Newsome et al., 1978). The structure of ST_b has been shown by nucleotide sequencing to be distinctly different from that of ST_a (Lee et al., 1983; Picken et al., 1983), and the two are also immunologically unrelated. The ST_a toxin has 2 subclasses (Moseley et al., 1980, 1983; So and McCarthy, 1980), 1 human (ST_h) and 1 porcine (ST_p), which show a high degree of homology (Aimoto et al., 1982; Chan and Giannella, 1981; So and McCarthy, 1980; Takao et al., 1983). The STp, also known as ST_{Ia}, is produced by porcine, bovine and human strains and ST_h, also known as ST_{Ib}, is produced only by human strains.

The ST_as of human, porcine and bovine origin have been purified, synthesized and sequenced (Aimoto et al., 1982; Chan and Giannella, 1981; Lallier et al., 1982; Lazure et al., 1983, Ronnberg et al., 1983; Saeed et al.,1983). The toxins consist of 18 or 19 amino acids (Lallier et al., 1982) which share common core sequences and immunological domains. Twelve amino acids appear to be conserved with respect to their positions in the toxins (Lazure et al., 1983; Staples et al., 1980).

The ST is considered to be nonantigenic, although it has been shown to act as a hapten. It may be made antigenic by coupling it to a carrier protein (Alderete and Robertson, 1978; Frantz and Robertson, 1981). According to Alderete and Robertson (1978), the toxin has a molecular weight of 4.4 KDa. Consisting of a small family of polypeptides, ST_a polypeptides share 13 common amino acids in the region of the active site (Aimoto et al., 1982; Chan and Giannella, 1981; Thompson et al., 1985). The ST_a toxin, which has a high content of half-cysteines (Alderete and Robertson, 1978), has 3 disulfide bonds, some of which are important for its biologic activity (Chan and Giannella, 1981; Dreyfus et al., 1983, 1984; Greenberg et al., 1983b).

The action of ST is almost instantaneous, relatively short lasting and readily reversible by rinsing (Evans et al., 1973; Field et al., 1978; Guerrant et al.; 1980; Hughes et al. 1978). In contrast to LT, the action of ST is primarily restricted to intestinal cells (Guerrant et al., 1980; Rao et al., 1980), but receptors for ST_a have also been found on rat basophil leukemia cells (Thomas and Knoop, 1983). The St_a toxin has a much more pronounced effect on the small intestine and cecum than the colon (Guerrant et al., 1980; Rao et al..; 1980). The variation in the effect on the intestine is probably due to the tissue distribution of the ST receptors, whose molecular nature is unknown. However, the ST_a toxin is known to bind to brush border membrane receptors (Frantz et al., 1984; Giannella et al., 1983). It has been reported that the number of receptors per microgram of membrane protein is greater in infants than in older children and that the number of receptors decreases rapidly with age (Cohen et al., 1988).

Utilizing various assay systems, St_a does not appear to activate adenyl cyclase (Hamilton _et al._, 1978; Sack and Sack 1975; Smith and Gyles, 1970). The mechanism of action of ST_a involves the activation of guanylate cyclase in small intestinal epithelial cells (Field _et al._, 1978; Hughes _et al._, 1978; Newsome _et al._, 1978b). Guanylate cyclase activity in the rat small intestine occurs mainly in the intestinal microvillus, with considerably greater activity at the differentiated villus tip than in the crypts (de Jonge, 1975). The ST_a toxin ultimately leads to a decrease in sodium chloride absorption and an increase in chloride secretion (Guandalini _et al._, 1982; Rao _et al._, 1980).

There are several postulated pathways for the activation of guanylate cyclase. The ST_a toxin may interact directly with the transmembrane guanylate cyclase receptor located in intestinal microvilli (de Jonge, 1975, 1984). Another suggested pathway involves the activation of a calcium/calmodulin-dependent phospholipase A_2 via ST_a to produce arachidonate. Arachidonate then proceeds by the cyclooxygenase or lipoxygenase pathways to produce a free radical that activates guanylate cyclase (Guerrant _et al._, 1980; Greenberg _et al._, 1980, 1982a; Knoop and Abbey, 1981; Knoop and Thomas, 1983). A report by Dreyfus _et al._ (1984) disputes this theory however.

Subsequent to the activation of guanylate cyclase, a protein kinase and calcium have been implicated in the events that lead to secretion. There are 2 specific cyclic guanosine monophosphate (cGMP)-dependent protein kinases. One, G-kinase, type II described by de Jonge (1984) is found only in intestinal microvilli. The G-kinase is an 86 KDa protein which can be split into 71 and 15 KDa fragments. The larger fragment binds cGMP and catalyzes phosphorylation of specific sites on proteins. The smaller fragment contains 1 of 2 cGMP-dependent phosphorylation sites present in the intestinal microvilli. This self-phosphorylation site may provide an efficient step in the events that lead rapidly to active chloride secretion after the addition of ST_a (de Jonge, 1981). The second cGMP-dependent phosphorylation site is on a 25 KDa protein that is also present on intestinal microvilli (de Jonge, 1984). This protein may be phosphorylated by either cAMP- or cGMP-dependent protein kinases. It is not known whether autophosphorylation of the 15 KDa fragment of the G-kinase or phosphorylation of the 25 KDa G-kinase substrate directly alters a nearby anion channel or whether cGMP, the G-kinase or the phosphorylated G-kinase substrate then subsequently changes calcium balance in the intestinal brush border to cause secretion. Some agents that affect calcium and calmodulin also affect secretory responses to ST or cGMP analogues (Guerrant et al., 1980; Greenberg et al., 1980, 1982a,b).

The ST_b toxin's mechanism of action is believed to be cyclic nucleotide independent (Kennedy et al., 1984). Like ST_a, ST_b has a rapid but reversible onset of action. In vivo, ST_b causes a significant increase in the amount of bicarbonate, sodium and chloride in the intestinal contents (Weikel et al., 1986a). The ST_b producing E. coli strains are not believed to be a major cause of diarrhea in people (Weikel et al., 1986b).

Bioassays used for the detection of ST_a include the suckling mouse assay (Dean et al., 1972) and the ligated pig loop assay (Smith and Gyles, 1970). Several other tests for the detection of ST_a have also been described, ELISA (de Mol et al., 1983, 1985, Klipstein et al., 1984; Lockwood and Robertson, 1984; Thompson et al., 1984), RIA (Dreyfus et al., 1983; Frantz and Robertson, 1981; Giannella et al., 1981) and DNA hybridization (Georges et al., 1983; Moseley et al., 1980; Seriwatana et al., 1983). Also used for the detection of ST_a is an in vitro assay which determines the activation of intestinal membrane guanylate cyclase (Waldman et al., 1984).

Innate Resistance to _E. coli_ Enteric Infections

Neonatal pigs are highly susceptible to diarrhea caused by ETEC. Several innate mechanisms of resistance exist within the pig's intestine to inhibit the successful colonization of the small intestine by ETEC (Newby and Stokes, 1984).

One of the first barriers that bacteria, such as _E. coli_, must face is gastric acid. The low pH of the stomach acts as a bactericidal trap which limits the number of viable bacteria and viruses which can enter the intestine. At a pH of 3.0 the bactericidal effect is complete (Gray and Shiner, 1967). The susceptibility of bacteria and viruses to low pH does vary, though. However, the pH of the neonatal pig's stomach is relatively high, it has been reported to be greater than 5.0 (Smith and Jones, 1963). Thus, in the neonatal pig more viable bacteria are able to enter the small intestine.

Gut peristalsis is another important factor which limits the number of bacteria within the small intestine. Organisms entering the small intestine are rapidly removed by peristalsis (Dixon, 1960). Peristaltic contractions and villous pumping act as major defense mechanisms in the small intestine and thus it is essential, as in the case of ETEC, that bacteria adhere to the small intestinal mucosa to overcome this clearance mechanism (Moon, 1980). Mucus is also capable of acting as a protective agent against bacteria in the small intestine. Flowing mucus and ingesta act to cleanse the epithelial surface (Moon, 1980). Mucus also entraps bacteria (Shrank and Verwey, 1976) which eases their removal. As mentioned previously, mucus contains substances that resemble epithelial receptor sites for bacteria, and these substances may also facilitate bacterial trapping (Strombeck and Harrold, 1974).

The normal microflora of the small intestine acts to resist colonization by pathogenic bacteria. Suggested mechanisms via which normal bacteria prevent colonization by pathogenic bacteria include competition for space, nutrients or receptor sites. Also, the normal microflora may make the environment toxic to the pathogenic bacteria by altering the pH or producing toxic substances such as volatile fatty acids and colicins (Mushin _et al._, 1970).

Substances present in the intestine, such as lactoferrin and lysozyme, also have antibacterial effects. Lactoferrin, which is present in intestinal secretions (Masson _et al._, 1966), successfully competes for iron with _E. coli_ and thus has a bacteriostatic effect on _E. coli_. Lysozyme, produced by the Paneth cells of the small intestine (Sandow and Whitehead, 1979), also has antibacterial effects. Interferon plays an important role in antiviral defense in the small intestine.

Immunoglobulins present in intestinal secretions play a role in the protection of mucosal surfaces from bacteria. The major antibody in porcine intestinal secretions is immunoglobulin A (IgA). Secretory IgA interferes with the binding of microorganisms and their products to epithelium. Thus, IgA could prevent diarrhea due to ETEC by inhibiting the adhesion of E. coli and the binding of its enterotoxins to intestinal epithelial cells. The Igs, IgG and IgM, may also play roles in the protection of the small intestine against bacteria (Newby and Stokes, 1984).

As pigs age, they become resistant to ETEC (Moon and Whipp, 1970; Nielsen et al., 1968; Smith and Halls,,1967). Diarrhea caused by K88-positive ETEC may occur during the neonatal and postweaning period, but diarrhea due to K99-positive ETEC is not known to occur after weaning in pigs (Moon, 1978b; Sojka, 1965). The mechanism of age resistance is unknown.

Some pigs may be born resistant to diarrhea caused by K88-positive ETEC (Gibbons et al., 1977; Sellwood et al., 1975). British researchers have determined that the presence or absence of receptors for the K88 pilus of E. coli is inherited. Two alleles are present at a single locus and are inherited in a simple Mendelian manner. One allele, the allele coding for the receptor, is dominant over the other allele. Three genotypes occur: homozygous dominant (SS, susceptible, adherent), heterozygous (Ss, susceptible, adherent) and homozygous recessive (ss, resistant, nonadherent). Two phenotypes occur, susceptible and resistant. The susceptible or positive phenotype is the expression of the dominant allele (Gibbons *et al.*, 1977; Sellwood *et al.*, 1975). Phenotypic expression is independent of the age of the pig and is fully established at birth (Gibbons *et al.*, 1977). Sellwood (1980a) has also described another phenotype as weak adhesive which is probably coded for by a third allele. With the weak adhesive phenotype, very few K88-positive bacteria adhere to the intestinal brush border *in vitro*, compared to the number of bacteria attached to brush borders of the original susceptible phenotype. The phenotype can be determined by using a simple *in vitro* technique described by Sellwood *et al.* (1975) which demonstrates the adhesion of K88-positive *E. coli* to brush borders from pig intestinal cells. Adhesion of bacteria to brush borders occurs in pigs which have the receptors (positive pigs) and no adhesion occurs in pigs lacking the receptors (negative pigs) (Sellwood *et al.*, 1975).

Rutter *et al.* (1973) have shown experimentally that negative pigs are resistant to infection by K88-positive *E. coli*. The K88-positive bacteria colonized the gut of positive pigs more readily than the gut of negative pigs, and the positive piglets were more likely to be susceptible to diarrhea caused by E. coli. In negative piglets, the organisms were unable to attach and rapidly disappeared from the intestines (Rutter et al., 1973). Sellwood (1979) reported that in a natural outbreak of scours, negative piglets were resistant to diarrhea caused by E. coli. However, susceptibility to infection is more complicated in a clinical situation. Positive piglets receiving colostrum or milk from the dam that contains K88 antibodies are passively protected (Rutter and Jones, 1973). It is assumed that positive piglets are susceptible to infection, but passive protection due to K88 antibodies in the colostrum prevents ETEC attaching and multiplying to high numbers in the small intestine. The negative phenotype primarily determines whether an animal will be resistant to infection with K88-positive strains of E. coli, but if the positive animals receive antibodies, they may also be resistant to infection (Rutter et al., 1973).

More recently, Bijlsma et al. (1982) in the Netherlands, found that there are 5 phenotypes in swine based on the 3 serologic variants of the K88 antigen. The phenotypes differ depending upon whether or not a pig is susceptible to adhesion in the brush border test to 3 (phenotype A), 2 (phenotype B and C) or 1 (phenotype D) of the serologic variants of the K88 pilus or resistant to all 3 variants (phenotype E). Sellwood's adhesion-negative phenotype corresponds to the D and E phenotypes, because the K88ad serologic variant of the *E. coli* was not recognized when he performed his study. The existence of 5 phenotypes complicates the rather simple genetic model proposed by British researchers, and additional work is needed to determine the inheritance pattern of the 5 phenotypes. Work by the Rapacz and Hasler-Rapacz (1986) suggests that phenotypes A and B correspond to 2 haplotypes with genes at 2 or 3 closely linked loci or that the phenotypes are expressions of alleles at 1 locus, with each allele specifying a receptor able to bind 2 or 3 different serological variants of the K88 pilus.

Moon *et al.* (1979) stated that they have not encountered pigs congenitally resistant to colonization by *E. coli* carrying the K99 or 987P pili. However, using isolated small intestinal epithelial cells from pigs 1 day, 3 weeks and 6 weeks old, Runnels *et al.* (1980) demonstrated resistance with age to K99-positive *E. coli*. From 8.8 to 14.5 more K99-positive ETEC were shown to adhere to 1-day-old piglet epithelial cells than to 6-week-old pig epithelial cells. The resistance to adhesion wasn't demonstrable with 3-week-old pigs.

Immunoprophylaxis

E. coli Vaccines

Both pili and enterotoxins are being used as immunogens for ETEC vaccine development.

Pilus Vaccines. Currently, several pilus vaccines have been developed for the protection of pigs against neonatal enteric colibacillosis. Pili act as important antigens for vaccine development because they are present on the bacterial surface, thus making them readily accessible to antibody and also because pilus production is an essential step in the early pathogenesis of enteric colibacillosis. Vaccines are administered parenterally or orally to sows prior to farrowing (Moon, 1981). The vaccine induces protective levels of antibody in the sow's colostrum and milk which provide passive immunity to the newborn pig. Research has demonstrated that protection correlates with antipilus antibodies in the colostrum and milk (Acres *et al.*, 1978; Isaacson *et al.*, 1980; Morgan *et al.*, 1978; Nagy *et al.*, 1978; Rutter and Jones, 1973; Rutter *et al.*, 1976). Several researchers believe that colostral antibodies prevent the colonization of ETEC by blocking adhesion to specific epithelial receptors (Nagy *et al.*, 1978; Rutter, 1975; Rutter and Jones, 1973; Rutter *et al.*, 1976). Without successful colonization, the ETEC are not able to produce a sufficient amount of enterotoxin to cause diarrhea. Piglets suckling vaccinated dams are more resistant to infection by ETEC than control pigs when challenged by ETEC with homologous pili (Moon, 1981). According to several researchers, the mortality, morbidity and the duration of diarrhea are decreased in piglets suckling vaccinated dams (Morgan *et al.*, 1978; Nagy *et al.*, 1978; Rutter *et al.*, 1976). Also, the number of *E. coli* attached to the villous epithelium of the small intestine is decreased (Nagy *et al.*, 1978).

Several vaccines which use either purified or semipurified pili (Acres *et al.*, 1979; Morgan *et al.*, 1978; Myers, 1978; Myers and Guinee, 1976; Myers *et al.*, 1973; Nagy *et al.*, 1985; Rutter and Jones, 1973; Sojka *et al.*, 1978) or whole bacterial cell preparations have been developed. Since pigs may be infected by strains of ETEC with different pilus antigens, multivalent vaccines are necessary to provide protection from enteric colibacillosis.

Initially, whole cell bacterins were used to protect pigs against enteric colibacillosis (Kohler, 1974; Kohler *et al.*, 1975; Rutter and Anderson, 1972; Wilson, 1974). Pitman-Moore has developed a trivalent bacterin called Porcimune which contains K88ac, K99 and 987P piliated strains of *E. coli* (Anonymous, 1981). Pitman-Moore has also developed an *E. coli* bacterin containing 4 piliated strains of *E. coli*, K88, K99, 987P and F41 (To, 1984a). Recently, a multiple pilus phase cloned whole cell bacterin containing K88ac, K99, 987P and type 1 pili has also been shown to be protective against ETEC under natural and laboratory conditions (Brinton et al., 1983; Jayappa et al., 1983, 1984).

Subunit vaccines, consisting of pili only, have also been developed. A trivalent subunit vaccine, containing K88, K99 and 987P pili, which utilizes recombinant DNA gene splicing techniques has been made by Salsbury. The plasmids responsible for pilus production are introduced into a laboratory strain of E. coli. The laboratory strain of E. coli then produces many more pili than are normally produced by a wild strain of E. coli. The pili are sheared from the bacterial surface by mechanical means and incorporated into the vaccine. The endotoxins associated with bacterins, which may cause adverse effects such as allergies, shock and abortions, are excluded from subunit vaccines (Anonymous, 1983). A subunit bacterin containing K88, K99, 987P and F41 pili has also been produced (Schlink, 1985).

Enterotoxin Vaccines. Toxoid vaccines have been developed against LT and ST. For an E. coli toxoid vaccine to provide complete protection against any strain of ETEC, regardless of its somatic serotype or pilus antigens, it should contain both LT and ST (Klipstein et al., 1982).

Piglets suckling sows parenterally immunized with porcine LT have been protected from ETEC diarrhea (Dobrescu and Huygelen, 1976; Dorner _et al._, 1980). A vaccine containing procholeragenoid, an attenuated form of cholera toxin, has also been shown to be protective against enteric colibacillosis (Frantz and Mellencamp, 1984; Furer _et al._, 1982, 1983a,b).

Vaccines for ST_a have also been developed. The ST_a enterotoxin is nonantigenic, but is capable of acting as a hapten and has therefore been conjugated to carrier proteins such as bovine serum albumin or Ig (Frantz and Robertson, 1981; Giannella _et al._, 1981). In a study by Moon _et al._ (1983) where pregnant swine were immunized with ST_a coupled to bovine Ig, suckling pigs were not protected against challenge with ETEC producing ST alone. The vaccine stimulated the production of antibodies with high binding activity but low neutralizing activity and thus low protective activity for suckling pigs. An experimental synthetic ST_a vaccine has been produced. However, protection from disease was only partial and not as effective as vaccination with whole cell bacterins (Frantz _et al._, 1987).

Immunization of animals with LT, its B subunit or ST provides protection against ETEC that produce homologous but not heterologous enterotoxins (Klipstein _et al._, 1983a,b). Therefore, experimentally, vaccines containing

ST chemically coupled to LT have also been developed (Klipstein *et al.*, 1982, 1983a,b). A completely synthetic toxoid vaccine containing ST and antigenic determinants of the LT toxin's B subunit has recently been developed (Houghten *et al.*, 1985).

Passive Immunization

Immunologically, the newborn pig is relatively defenseless and thus highly susceptible to infection. Passive immunization, via the absorption of antibodies from colostrum and milk, of the neonatal pig prior to its exposure to an infectious agent protects the pig from infection. The correlation of the 3 ages of peak incidence of porcine enteric colibacillosis, neonatal, 3 weeks and immediately postweaning, with periods of antibody deficiency indicate that such a deficiency predisposes pigs to ETEC (Moon, 1974). Since adhesion of ETEC to the intestine is an essential prerequisite for the development of enteric colibacillosis, antibody-mediated prevention of bacterial adhesion is a potential defense mechanism against *E. coli*. Such antibody may be acquired from colostrum and milk.

Serum, Colostrum and Milk. There have been several reports in the literature demonstrating the effectiveness of hyperimmune serum, colostrum and milk against experimental challenge with ETEC. The predominant Ig in porcine serum and colostrum is IgG, however IgA is the predominant Ig in milk and intestinal secretions (Porter, 1969a,b; Porter *et al.*, 1970a,b; Curtis and Bourne, 1971; Bourne, 1976).

As early as 1966, Kohler and Bohl showed that orally administered serum, obtained from hyperimmunized gnotobiotic pigs, had a protective effect against *E. coli* in infected pigs. Four- to 6-day-old gnotobiotic pigs were fed 7 ml of hyperimmune serum in milk one and a half hours prior to challenge. The serum in milk was then administered every one and a half hours for 30 to 36 hours. No clinical signs of diarrhea were observed during the period the serum was administered. However, the immune serum only had a temporary protective effect; 12 to 24 hours after the serum was last given, diarrhea ensued. Heated antiserum was administered in a similar manner with similar effects. Three and a half milliliters of immune serum in milk were also administered to a few pigs during a 24 hour period with a comparable temporary protective effect. Results indicated that the hyperimmune serum's protective action was apparently not dependent upon the complement-antibody bactericidal system, but was thought at that time to be related to the inactivation of toxin in the intestinal lumen.

In another study by Kohler (1967), the effects of parenterally, as well as orally, administered antisera, produced in conventional swine, were evaluated in gnotobiotic pigs infected with E. coli. Not only were the animals observed clinically, but gross, histologic and microbiologic observations were also made. The protocol for oral administration of antiserum was the same as that previously described (Kohler and Bohl, 1966). For the parenteral administration of antiserum, 14 ml of antiserum were injected intraperitoneally 16-20 hours prior to oral infection. Pigs were killed at predetermined time intervals between 6 and 48 hours postinfection. Orally administered antisera protected pigs from fluid loss and diarrhea. With the parenterally administered serum, diarrhea was not observed, but there was fluid loss into the intestinal lumen. The mechanism of protection did not appear to involve a marked reduction in the number of live E. coli in the small intestine.

In 1968, Rejnek et al. fed colostrum and serum from immunized sows to gnotobiotic pigs infected with the homologous E. coli strain. Two hours after challenge, 3-day-old pigs were given 12.5-50 ml immune colostrum or serum. Control pigs were given serum from nonimmunized sows. Pigs fed colostrum or serum from immunized sows survived whereas the controls died.

Miniats et al. (1970) attempted to protect pigs against enteric colibacillosis by orally administering E. coli antisera from immunized specific pathogen free pigs. Six-day-old gnotobiotic pigs were given 4 ml of antiserum or normal serum 30 minutes prior to challenge with homologous *E. coli*. Thereafter, the serum was administered at 8 hour intervals for 3 consecutive days. Antiserum directed against 1 strain of K88ab-positive *E. coli* used was demonstrated to have a protective effect. The 3 pigs receiving specific antiserum against the homologous strain of *E. coli* with which they were infected remained healthy while the serum was administered, but 2 of the 3 pigs died 2 days following the withdrawal of the serum. Antiserum against the K88ab-positive strain of *E. coli* did not significantly decrease the number of *E. coli* present in the intestine between treated and control animals, suggesting that the protective effect of the antibody in the intestine was due to its action on the enterotoxin.

Svendsen and Wilson (1971) observed that feeding colostral whey or serum from sows intramammarily or intramuscularly vaccinated with a live formalin-attenuated K88ac-positive *E. coli* vaccine to gnotobiotic pigs infected with *E. coli* was protective, as demonstrated by an increased survival time. Pigs given the colostral whey from vaccinated sows also had a delayed onset of diarrhea. However, those receiving colostrum from nonvaccinated sows did not. Ten-day-old gnotobiotic pigs were fed colostrum or serum (10 or 40 ml) 6 hours prior to challenge and at 8 hour intervals following the first feeding for 3 days.

Diarrhea appeared in all infected pigs except those treated with colostrum from sows vaccinated by the intramuscular route, in which it was not observed until 24 hours after the cessation of colostrum feeding. Results indicated that in order for antibody to be effective, it must be continually present in the intestinal tract of pigs. In an associated study, whey prepared from milk collected 7 days after farrowing increased the survival time but did not delay the onset of diarrhea in infected gnotobiotic pigs (Wilson and Svendsen, 1971). Control pigs were given milk whey from nonvaccinated sows or condensed milk. The protocol was similar to the previous experiment. All the pigs developed profuse diarrhea 8 to 16 hours after infection, and the diarrhea continued throughout the experiment with no difference in the severity of diarrhea or the rapidity of onset of dehydration between pigs in the different treatment (10 or 40 ml) groups. The pigs fed 40 ml of milk whey from vaccinated sows showed an increased survival time. In another study by Wilson (1972), colostrum from immunized sows or IgG isolated from that colostrum was orally administered to gnotobiotic pigs. Based on a prolonged survival time, significant protection of the pigs from enteric colibacillosis was reported.

Smith and Linggood (1971b) parenterally administered 125 to 200 ml of E. coli antiserum, depending upon the size of the pig, to conventionally reared weaned pigs. The administration of antiserum usually prevented the pigs from developing diarrhea or edema disease after infection with K88-positive _E. coli_. Protection was only demonstrated when animals were infected with the homologous strain of _E. coli_, and protection appeared to correlate with a failure of the infecting organism to proliferate in the intestine. Protection was believed to be primarily bactericidal, but an antienterotoxic effect was also suggested. In a study by Smith (1972), colostrum-deprived pigs were given 20 ml of antiserum in milk orally, and then 2 hours later the pigs were infected orally with K88-positive ETEC and a larger number of nonpathogenic _E. coli_ and lactobacilli. Controls were given normal serum. Results of the study indicated that antiserum prepared against live, heat-killed or methanol-killed bacteria of the 0141:K85ab, 88ab strain inhibited the multiplication of homologous ETEC in the small intestine of pigs given the antiserum. This effect, considered to be antibacterial, was felt to be responsible for delaying or preventing the onset of diarrhea. An antienterotoxic effect didn't appear to be present since antiserum against the enterotoxin did not delay the onset of diarrhea caused by _E. coli_ 0141:K85ab, 88ab. Antisera against the O or other K antigens of the strain 0141 also had no effect. However, antisera against the pathogenic

E. coli strain 08:K87, 88ab had a diarrhea-controlling effect, apparently attributable to the K88ab antigen common to both strains of *E. coli*. Smith postulated that the protective effect was associated with the inhibition of adhesion by the K88 antigen of *E. coli*. Antiserum was also administered subcutaneously, it had a controlling effect on diarrhea but it was less effective than antiserum administered orally. Parenteral therapy with serum has indicated that antibody can pass into the intestinal tract of pigs and possibly contribute to local immunity (Murray, 1973). In a study by Morilla *et al.* (1984) serum from adult pigs was given orally to neonatal pigs 8 hours after birth. The treated animals had a better appearance, a higher body weight, more stamina and less diarrhea than the controls.

Recently, Bar-Guard-99, an oral calf serum from Anchor, has been used commercially for the prevention of enteric colibacillosis in calves. Bar-Guard-99 contains whole cell antibodies to K99-positive *E. coli* and thus provides polyclonal protection against enteric colibacillosis. A company trial indicated that the survival rate of animals receiving Bar-Guard-99 was twice that of animals receiving a K99 MCA product (Anonymous, 1987).

In vitro, the Fab fragments of antibody specific for 987P pili have been shown to block the adhesion of 987P-positive ETEC to pig small intestinal cells (Isaacson *et al.*, 1978). Also using intestinal epithelial cells, Wilson and Hohman (1974) inhibited the adhesion of *E. coli* bearing the K88ab or K88ac antigen with homologous antisera against the K88 b or c antigens. Antisera against K88-positive *E. coli* also inhibited the adhesion of *E. coli* to disks of porcine intestinal mucosa obtained from gnotobiotic pigs (Jones and Rutter, 1972). Parry and Porter (1978) were able to block the adhesion of K88ab- and K88ac-positive strains of *E. coli* to brush borders by using antisera specific for the K88a determinant. Antibodies against K88b and K88c antigens only inhibited homologous strains of K88-positive *E. coli*.

Colostrum or milk from sows vaccinated orally with live cultures of *E. coli* also protected gnotobiotic piglets against diarrhea when challenged with the homologous ETEC (Kohler, 1974). The pigs were fed colostrum or milk, infected 26 hours after birth and then were continuously fed milk or colostrum till 48 hours of age. Results of preliminary experiments indicated that better protection was provided pigs fed mammary secretions from the first feeding than was provided by pigs initially fed the mammary secretions only 3 hours before infection. The colostrum and milk inhibited the multiplication of a large number of both the homologous

K88-positive and homologous K88-negative strains on the intestinal mucosa.

Scoot et al. (1972) gave gamma globulins orally to piglets. When the globulins were given for only 1 day, the piglets' serum IgGs rose to near normal levels, but the piglets died of colibacillosis. However, when they were given gamma globulins orally for a minimum of 10 days, colibacillosis did not occur. Miler et al. (1975) determined the protective effect of porcine colostrum, serum, IgG, IgM and IgA against an enterotoxic strain of E. coli in newborn germfree pigs. They reported that IgA isolated from immune porcine sera and colostrum was effective at a lower concentration against ETEC in ligated pig loops than IgG or IgM isolated from the same sources. It has been reported that the protective effect of human milk against gastrointestinal illness in infants (Chandra, 1979; Cunningham, 1979; Gerrard, 1974; Jason et al., 1984; Kovar et al., 1984; Larsen and Homer, 1978) may be due to specific antibodies (Glass et al., 1983; Majumdar and Ghose, 1982; Stoliar et al., 1976) as well non-immunoglobulin components (Goldman and Smith, 1973; Holmgren et al., 1983; Welsh and May, 1979).

In vitro studies have also demonstrated the anti-adhesive activity of colostrum and milk. In a study by Rutter et al. (1976) colostral antibody inhibited the adhesion of K88-positive E. coli to slices of intestinal tissue. Antibody from sow's milk has been shown to inhibit the attachment of K88-positive *E. coli* to pig intestinal epithelial cells (Evans *et al.*, 1980). Using pigs from a herd which had acquired natural immunity to K88-positive *E. coli*, Sellwood (1984b) reported that colostrum from genetically susceptible sows in the herd inhibited the binding of radiolabelled K88 antigens to brush borders better than colostrum from genetically resistant dams. The colostrum from susceptible dams was also more efficient in *in vitro* opsonic phagocytosis and killing of K88-positive *E. coli* than colostrum from resistant sows. Also, antibodies from sheep colostrum inhibited the attachment of K99-positive *E. coli* to sheep intestinal epithelial cells (Morris *et al.*, 1980b).

Monoclonal Antibody. Recently, an alternative method for the direct passive immunization of newborn animals against ETEC, the oral administration of *E. coli* pilus-specific, hybridoma-derived MCA, has been developed. Since Kohler and Milstein (1975) first produced hybridomas, there have been amazing advances in biomedical research, immunodiagnostics and immunotherapy. In the area of infectious diseases, MCAs have been used to determine antigenic structure, virulence mechanisms, and host responses to a wide variety of bacteria, viruses and parasites. Numerous advances in immunodiagnosis, prophylaxis and therapy have resulted. A recent review by

Sherman and Markham (1986) describes the current and future applications of MCAs against bacteria in veterinary medicine.

Disease prevention by passive immunization is one possible clinical use of MCAs in veterinary medicine. Monoclonal antibody therapy might be of particular benefit when unexpected disease outbreaks occur in susceptible herds or flocks where a vaccination program has not been undertaken, effective vaccines are not available or the cost-benefit ratio of prophylaxis was not considered (Sherman and Markham, 1986). Recently, there have been several reports in the literature concerning the passive immunization of neonatal animals against enteric colibacillosis.

One of the first reports involved the oral administration of _E. coli_ K99 MCA to calves (Sherman _et al._, 1983). Since calves and lambs are susceptible to K99-positive ETEC only during the first few days of life (Runnels _et al._, 1980; Smith and Halls, 1967) it was assumed that, if antibody against the K99 pilus was present in the intestine during this time, colonization and thus diarrhea could be prevented until the animal became naturally resistant to infection by ETEC. Three separate challenge trials were used to determine the efficacy of the MCA. In the first trial, newborn calves were fed colostrum, treated with 1 ml of a 1:12,000 titer

K99 MCA at 10 hours of age and then challenged with K99-positive *E. coli* at 12 hours of age. The protocol in the other 2 trials was similar except that the calves were colostrum deprived. In the study, there was a statistically significant reduction in the mortality rate and the severity and duration of diarrhea in the treated calves versus the control calves. However, there was no difference in the incidence of diarrhea. Several explanations were postulated for the diarrhea which occurred in animals which received the K99 MCA. It was speculated that the amount of MCA used may not have completely blocked colonization. Also, it was suggested that the number of bacteria in the challenge inoculum might have been large enough to produce sufficient enterotoxin, without adhesion to the mucosa, to cause diarrhea. The previous study led to the development of a commercially available K99 MCA for calves, Genecol 99 (Molecular Genetics, Inc. Minnetonka, MN).

Using Caesarian-derived, colostrum-deprived piglets, the oral administration of K99 MCAs has also been shown to be protective against challenge with K99-positive ETEC. Pigs were fed 100 mg of MCA and then challenged with 1 x10^{10} K99-positive *E. coli*. Eleven of 14 pigs (79%) which received the MCAs survived the challenge in comparison to only 2 of 10 survivors (20%) in the unprotected group (Sadowski *et al.*, 1983). In a similar study, newborn calves were protected against challenge with K99-positive *E. coli* by the oral administration of 20 mg of K99 MCA. Six of 7 (86%) treated calves survived compared to 2 of 8 (25%) survivors in the control group (Sadowski *et al.*, 1983). In another study, 8 hysterectomy derived, colostrum-deprived pigs were used; 4 pigs received K99 MCA and 4 received K88 MCA. The pigs received the MCA 3 times a day mixed with milk replacer from 2 through 7 days of age and were observed for 17 days after challenge. The pigs were challenged with a K99-positive strain of *E. coli* just before their second MCA feeding at 2 days of age. All the challenged pigs developed diarrhea and 1 pig in each group died. The diarrhea started later and ended earlier in the pigs fed K99 MCA compared to those fed K88 MCA. The mean concentration of available K99 antigen in the feces of pigs fed K99 MCA was lower than in those receiving K88 MCA in the initial days of the study (Mainil *et al.*, 1987). The K99 MCA used in all the previously-described studies was obtained from the same hybridoma.

Neonatal, colostrum deprived pigs have been protected from fatal colibacillosis due to K88-positive ETEC through the use of "a" and "c" specific MCAs to the K88 pilus (Sadowski, 1984). In an experimental trial, a K88 MCA against all 3 serologic variants was orally administered to newborn pigs. A therapeutic effect was noted, but there was no prophylactic effect. Pigs given a therapeutic treatment with the MCA (29 pigs), with or without a preceding prophylactic treatment, had a zero mortality rate. However, in 1 litter, where the antibody was only administered prophylactically, all of the animals died. In the control group, which was not treated, 9 of 12 animals died (Foged *et al.* , 1986).

A 987P MCA has also been used experimentally for the passive immunization of colostrum-deprived neonatal pigs. Seven of 11 treated animals survived challenge (64%) with 987P-positive *E. coli* compared to 1 of 11 (9%) of the control animals. The 987P MCA was also used in a field outbreak of enteric colibacillosis caused by 987P-positive *E. coli*. Ten litters were equally divided between placebo and control animals. The placebo treated animals had a higher mortality rate, 24 of 44 pigs (54%), than the treated animals, 8 of 44 (18%). The 987P pilus levels in the feces of the MCA-treated animals was lower than in the control animals (Sadowski *et al.*, 1986).

Molecular Genetics has evaluated the efficacy of a trivalent MCA mixture containing antibodies against K88, K99 and 987P pili against porcine enteric colibacillosis (Sherman and Markham, 1986).

CHAPTER 1

THE EFFECT OF 987P MONOCLONAL ANTIBODY ON THE ADHESION OF 987P-POSITIVE *ESCHERICHIA COLI* TO PIG INTESTINAL BRUSH BORDERS

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INTRODUCTION

Adhesion to the small intestinal mucosa is an essential prerequisite for the development of diarrhea due to ETEC in animals. Current knowledge indicates that pili present on the bacterial surface allow *E. coli* to attach to specific receptors present on the small intestinal mucosa. Receptors for the various pili present on strains of ETEC differ. *In vivo*, antibody, both naturally acquired and passively acquired through colostrum and milk from pilus vaccinated dams, has been shown to be protective against experimental and clinical ETEC infections. It is theorized that pilus specific antibodies prevent piliated bacteria from attaching to the small intestinal mucosa, thereby preventing subsequent steps in the pathogenesis of enteric colibacillosis. Evidence supporting the antiadhesive effect of pilus-specific antibodies has been demonstrated *in vitro* utilizing various assay systems.

Several *in vitro* techniques have been described which demonstrate the adhesion of piliated *E. coli* to the small intestine, as well as to oral epithelial cells and erythrocytes. Examples of the various methods are the brush border technique (Sellwood *et al.*, 1975), the intestinal epithelial cell technique (Isaacson *et al.*, 1978; Nagy *et al.*, 1977; Wilson and Hohman, 1974), the intestinal villous technique (Girardeau, 1980), the immobilized intestinal mucosa technique (Laux *et al.*, 1986), the buccal epithelial cell technique (Gibbons and van Houte, 1971), an indirect enzyme linked immunosorbent assay (Schifferli *et al.*, 1987) and hemagglutination assays (Evans *et al.*, 1979).

Several researchers have demonstrated that the *in vitro* adhesion of piliated *E. coli* to the small intestinal mucosa can be inhibited by antipilus antibodies in colostrum and milk. In a study by Rutter *et al.* (1976), colostral antibody inhibited the adhesion of K88-positive *E. coli* to discs of intestinal tissue. Antibody from sow's milk has been shown to inhibit the attachment of K88-positive *E. coli* to pig intestinal epithelial cells (Evans *et al.*, 1980). Using pigs from a herd which had acquired natural immunity to K88-positive *E. coli*, Sellwood (1980a) reported that colostrum from genetically susceptible sows in the herd inhibited the binding of radiolabelled K88 antigens to brush borders better that colostrum from genetically resistant dams. At concentrations of > 1mg/ml, colostral fractions from susceptible sows containing IgM or IgA caused almost 100% inhibition of adhesion, and fractions containing IgG were less effective in inhibiting adhesion. In a later study by Sellwood (1984b), IgG did not appear to act as an antiadhesin. The colostrum from susceptible dams was also more efficient in *in vitro* opsonic phagocytosis and killing of K88-positive *E. coli* than colostrum from resistant sows (Sellwood, 1980a). Also, antibodies from sheep colostrum inhibited the attachment of K99-positive *E. coli* to sheep intestinal epithelial cells (Morris *et al.*, 1980b).

Antisera have been used to prevent *in vitro* adhesion. Jones and Rutter (1972) used antiserum against K88-positive *E. coli* to inhibit the adhesion of *E. coli* to disks of porcine intestinal mucosa obtained from gnotobiotic pigs. The Fab fragments of antibody specific for 987P pili have been shown to block the adhesion of 987P-positive ETEC to pig small intestinal cells when the bacteria were preincubated with the Fab fragments (Isaacson *et al.*, 1978).

Using intestinal epithelial cells, Wilson and Hohman (1974) inhibited the adhesion of *E. coli* bearing the K88ab or K88ac antigen with homologous antiserum against the K88 b or c antigens. Rabbit antiserum was preincubated for 30 minutes at 37 °C with the K88-positive bacteria prior to the addition of the enterocytes. No inhibition of adhesion was seen with heterologous K88 antiserum or with K99 antiserum. Parry and Porter (1978) were able to block the adhesion of K88ab- and K88ac-positive strains of E. coli to brush borders by using antisera specific for the K88a determinant. Antibodies against K88b and K88c antigens only inhibited homologous strains of K88-positive E. coli. Using rabbit K88 antiserum, Sellwood et al. (1975) also demonstrated that adhesion of K88-positive E. coli to brush borders could be inhibited by the addition of rabbit antisera against the K88 pilus. Several dilutions of the antisera were preincubated with the bacteria at room temperature for 1 hour prior to the addition of the brush borders.

Antisera directed against type 1 pili of E. coli have been shown to inhibit attachment of E. coli to mammalian cells in vitro (Isaacson et al., 1978; Abraham et al., 1983; Guerina et al., 1983; Salit and Gotschlich, 1977; Silverblatt and Cohen, 1979; Silverblatt et al., 1982). Antiserum to E. coli mannose-sensitive pili inhibited the attachment of piliated bacteria to buccal epithelial cells (Weinstein and Silverblatt, 1983). It was assumed that the antibody inhibited adhesion by obstructing the mannose-specific binding domains of the pili. However, it was also suggested that the anti-pilus antibody may not have directly inhibited adhesion but may have reduced the number of potentially adhering bacteria by agglutination. Adherence was also prevented by monovalent Fab' fragments of IgG, and monovalent antibody fragments can bind to pili but are unable to cause agglutination. Also in this study, the inhibition of bacterial adherence was not augmented by complement-dependent bacteriolysis, opsonophagocytosis or intravascular clearance. Using antiserum prepared against type 1 pili Isaacson _et al._ (1978) inhibited the attachment of _E. coli_ to porcine intestinal epithelial cells.

In a study by Abraham _et al._ (1983), MCAs to type 1 pili were shown to inhibit the attachment of type 1 pili _in vitro_ to oral epithelial cells, guinea pig erythrocytes and yeast cells. One of the MCAs was quaternary structure-specific, only recognizing polymers of $\geq$ 6 subunits. The other MCAs were subunit-specific and were directed toward antigenic determinants "hidden" in the quaternary structural conformation of the intact pili. Disassociation of the pili into subunits exposed the inaccessible determinants but eliminated the quaternary structural determinants. However, reassembly of the subunits _in vitro_ restored the quaternary structural determinants but concealed the subunit determinants.

Only the quaternary structure-specific MCAs inhibited the adhesive properties of the pili to the cells *in vitro*.

In a study by Schifferli *et al.* (1987), an indirect ELISA adhesion assay was used to demonstrate the antiadhesive ability of 987P MCAs directed against various antigenic determinants of the pili. Three MCAs recognized exposed antigenic determinants present on intact pili. Two of the 3 antibodies demonstrated quaternary structural specificities, but one of the MCAs recognized assembled pili and individual pilus subunits. A fourth MCA was subunit-specific because it did not react with fully assembled pili. Piliated 987P-positive *E. coli* were preincubated for 1 hour with 987P MCAs at their "lowest subagglutinating titer". The preincubated bacteria were then allowed to adhere to ileal and jejunal epithelial cells, isolated from 1- to 2-week-old pigs, immobilized in microtiter plate wells. Bacterial adhesion was then determined by the ELISA. Only antibodies that bound to intact pili were able to completely block 987P-mediated adhesion to piglet epithelial cells, suggesting that this characteristic may be a prerequisite to successfully prevent adhesion. In the case of 987P pili and type 1 pili, it appears that MCAs directed against pili must recognize quaternary structural antigenic determinants in order for the MCAs to successfully prevent adhesion.

Interestingly, the type 1 pili and 987P pili have similar helical structural conformations.

The objective of this study was to determine if the *in vitro* adhesion of 987P-positive *E. coli* to pig intestinal brush borders could be prevented by the administration of *E. coli* 987P MCA.

MATERIALS AND METHODS

Specimens

Samples of ileum, each 6 to 8 inches in length, were obtained from 17 freshly killed slaughter-weight pigs at Michigan State University Meats Laboratory, East Lansing, MI. Additional ileal samples, 12 inches in length, were obtained from twelve 3-12-day-old pigs which were euthanatized with sodium pentobarbital[a]. Ileal samples were carefully flushed with cold 0.15 M NaCl solution to remove mucus and ingesta. They were then placed in plastic bags and packed in crushed ice until further procedures were carried out, usually within an hour of the time of slaughter.

Brush Border Preparations

Intestinal specimens were processed using a modification of the procedure described by Sellwood *et al.* (1975). In the laboratory, the intestinal specimen was

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[a] Butler Co., Columbus, OH.

ligated on one end, and the lumen was filled with an ethylenediaminetetracetate (EDTA) buffer solution (pH 6.8) until slight distention occurred. The other end of the intestine was then clamped with a hemostat. The buffer was at room temperature. To prevent drying of the external surface, sections of ileum were immersed in a similar solution containing 0.3 M sucrose instead of EDTA, (pH 6.8) for 15-20 minutes at room temperature. Next, the EDTA buffer in the intestinal lumen was discarded and replaced with the sucrose buffer until the lumen was half-filled. The epithelial cells were detached by gently massaging each intestinal section between the thumb and fingers. The intestinal contents were collected in 50 ml plastic centrifuge tubes. The intestine was again half-filled with sucrose buffer and massaged. The latter procedure was repeated until 25-40 ml of the epithelial cell suspension were obtained.

The following steps were performed at 4°C. The epithelial cell suspension was centrifuged at 1200 x g for 15 minutes[b]. The supernatant was removed from each tube, and the pellet was resuspended to a volume of 45-50 ml with cold 0.005 M EDTA (pH 7.4, adjusted with 0.5 M Na_2CO_3). Next, the suspension was homogenized with a Teflon serrated-tipped tissue grinder (clearance - 0.15-

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[b] International Refrigerated Centrifuge, Model Pr-6, International Equipment Co., Needham, MA.

0.23 mm)[c] by moving the pestle up and down 6 times while it rotated at approximately 900 rpm. The suspension was centrifuged at 300 x g for 5 minutes, and the supernatant was discarded. The pellet was resuspended in the EDTA buffer, then homogenization and centrifugation were repeated 3 to 5 times until the supernatant appeared clear. Next, the pellet was washed 3 times with Krebs-Henseleit (KH) buffer (pH 7.4). It was then resuspended to 8 times its volume with KH buffer and filtered through glass wool. The KH solution containing the brush borders was allowed to stand for 15 minutes to let any mucus present rise to the surface. The mucus was then discarded. For prolonged storage, part of the brush border suspension was mixed with an equal volume of glycerol and stored at -70°C (Bijlsma *et al.*, 1982).

Protein Assay

A modified Lowry protein assay (Lowry *et al.*, 1951) was performed to determine the amount of protein present in the brush border preparations. Bovine serum albumin (BSA)[d] at a concentration of 1 mg/ml was used as a standard. Using 13 x 100 mm glass tubes, 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg protein were added to individual tubes. Distilled, deionized water was added to

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[c] Thomas Co., Philadelphia, PA.

[d] Sigma Chemical Co., St. Louis, MO.

each of the tubes to give a total volume of 3.0 ml. Next, 20 μl of 2.5% sodium deoxycholate was added to each of the tubes, and the solution was well mixed. The tubes were then allowed to stand for 15 minutes. One milliliter of 24% trichloroacetic acid (TCA) was then mixed in each tube. The tubes were then centrifuged for 30 minutes at 3300 x g[e]. The Lowry protein assay was performed on the supernatant.

Using 12 x 75 mm glass tubes, 100 μl of 1N NaOH and 100 μl of distilled, deionized water were added to each standard sample. Distilled, deionized water was added to 25 μl and 50 μl samples of brush border suspension to produce a final volume of 100 μl. Next, 100 μl of 1N NaOH was mixed into each of the brush border samples. One milliliter of a freshly made solution, containing 98 parts 2% Na_2CO_3, 1 part 2% sodium tartrate and 1 part 1% $CuSO_4$, was added to each of the tubes. The tubes were then allowed to stand for 10-15 minutes. Next, 100 μl of phenol reagent[f], diluted 1:1 with water, were added to each of the tubes. The phenol reagent mixture, which is light sensitive, was covered with foil while being aliquoted. The tubes were then allowed to incubate and the optical density of each tube was read in a

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[e] Beckman Model J-6B Refrigerated Centrifuge, Beckman Instruments Inc., Palo Alto, CA.

[f] Harleco, Gibbstown, NJ.

spectrophotometer[g] set at 700 nm exactly 30 minutes after the addition of phenol reagent to that tube.

E. coli Strains

The 987P-positive *E. coli* used in the brush border adhesion test was strain 81-1469. The culture, obtained from Dr. David Francis, Department of Veterinary Science, South Dakota State University, Brookings, SD, was maintained in Minca broth (Guinee *et al.*, 1977) with IsoVitaleX[h] (MIB). Cultures of *E. coli* were aerobically incubated at 37°C in a shaking water bath[i] for 12-16 hours prior to testing for adhesion. The K88ac-positive strain of *E. coli*, strain I 248 (0157:K?), was obtained from Dr. R.E. Isaacson, School of Public Health, University of Michigan, Ann Arbor, MI and was grown in a similar manner.

Following incubation, both cultures were centrifuged at 1500 x g for 20 minutes. The supernatant was discarded, and the bacteria were washed in KH and centrifuged 3 times. The pellets were then resuspended to an optical

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[g] Beckman Spectrophotometer Acta III, Beckman Instruments Inc., Palo Alto, CA.

[h] Baltimore Biological Laboratories, Cockeysville, MD.

[i] Precision Scientific Co., Chicago, IL.

density between .075 and .085 at 560 nm[j]. The suspensions contained approximately 10^8 colony-forming units per ml.

Slide Agglutination

A standard slide agglutination test was carried out to verify piliation using 987P and K88 antisera. Prior to this study, the antiserum was produced in Dr. G.L. Waxler's laboratory, Department of Pathology, Michigan State University, East Lansing, MI. The antiserum was produced by hyperimmunizing rabbits with purified pili obtained from Dr. R.E. Isaacson. A 1:10 dilution of 987P or K88 antiserum was used for the tests. One drop of each of the bacterial suspensions was placed on a glass slide, a drop of antiserum was added, and the 2 drops were mixed with a wooden applicator stick. The glass slide was held near a light source with a magnifying mirror and slowly rocked and tilted in a circular motion for approximately 1 minute. Degrees of agglutination were recorded as strong, intermediate, weak or negative. Only those _E. coli_ cultures showing strong or intermediate agglutination were used in the brush border adhesion test. A control slide using KH instead of antiserum was used to check each of the bacterial suspensions for autoagglutination.

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[j] Shimadzu UV Visible Recording Spectrophotometer, Model UV-260, Shimadzu Corporation, Kyoto, Japan.

Monoclonal Antibody Production

A 987P hybridoma, 4A3, was used in the production of 987P MCAs of the isotype IgG_1. Prior to this experiment, the hybridoma was produced at South Dakota State University in Dr. David A. Benfield's laboratory according to the method of Greenberg et al. (1983a). Briefly, myeloma cells from the NS-1 cell line were fused with spleen cells from Balb/c mice immunized with the 987P pilus antigen. The NS-1 myeloma cell line is a non-secreting clone of P3X63 Ag 8 myeloma and produces Kappa light chains (Kohler and Milstein, 1975).

The hybridomas were grown as stationary suspension cultures in Dulbecco's Modified Eagle Medium containing L-glutamine and high glucose[k] (850 ml/l) supplemented with 10% heat-inactivated fetal bovine serum[d], 0.45 mM/ml sodium pyruvate[d] (10 ml/l), 100 units/ml penicillin G[k] (5 ml/l), 100 µg/ml streptomycin[k] (5 ml/l), bicarbonate (pH 7.5) (20 ml/l) and HEPES[d] (10 ml/l). Aseptic technique was used at all times when medium and cultures were being handled. The medium was prepared in a horizontal laminar flow hood and filtered prior to its use with a 20 µ disposable filter[l]. Polystyrene tissue culture flasks[m]

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[k] GIBCO, Grand Island, NY

[l] Nalge Co., Rochester, NY.

containing the cell suspension were incubated in an automatic CO_2 incubator[n] at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Cultures were examined using an inverted phase contrast microscope[o]. Under the laminar flow hood, the cell suspensions were split in half and then an equal volume of fresh medium was transferred aseptically to the culture flasks every other day. Cultures which were to be harvested were allowed to grow for 7 days without a change of medium and were then centrifuged for 10 minutes at 1000 x g[p] and the supernatant collected. The supernatant was then stored at 20°C. The specificity and titer of the antibody in the supernatant was checked at least once every 2 weeks using a bacterial slide agglutination test with 987P-positive *E. coli*.

When a sufficient volume of supernatant was collected (approximately 1 liter) antibody in the supernatant was concentrated by an ammonium sulfate precipitation. A saturated solution of ammonium sulfate was prepared by adding approximately 1 Kg $(NH_4)_2SO_4$ crystals per liter of

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[m] Corning Glass Works, Corning, NY.

[n] Queue Systems Dual Chamber Incubator, Model 2220, Parkersburg, West Virginia

[o] Model 1810, American Optical Corporation, Buffalo, NY.

[p] Model HN-S Centrifuge, International Equipment Co., Needham Heights, MA.

distilled water. The solution was stirred at room temperature for 8 hours. When most of the crystals were dissolved, the solution was stored overnight at 4°C. The pH was then adjusted to 7.0 with 30% (weight/volume) NaOH.

The saturated ammonium sulfate solution was used to precipitate the MCAs from the tissue-culture medium. An equal volume of saturated ammonium sulphate solution was added to the tissue culture supernatant. The mixture was mixed immediately by swirling, and precipitation was allowed to occur for 1 hour. The entire procedure was carried out on ice at 4°C. The precipitate was collected by centrifugation at 48,000 x g for 30 minutes[q]. After centrifugation, the supernatant was discarded and the precipitate was dissolved in 0.14 M sodium phosphate (pH 8.0). Using cellulose dialysis tubing with a molecular weight cut off of 12,000 to 14,000[r], the solution was dialyzed with 3 changes of 0.14 M sodium phosphate buffer (pH 8.0). The antibody titer was then checked using a slide agglutination test.

The MCA was further concentrated and purified using an affinity column chromatography procedure described by Ey <u>et al.</u> (1978). Protein A-Sepharose CL-4B[k] was swollen in 10 mM phosphate buffered saline (PBS) (pH 8.0)

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[q] Beckman L2-65B Ultracentrifuge (SW27 rotor), Beckman Instruments Inc., Palo Alto, CA.

[r] Spectra/Por 2 Dialysis Membranes, Spectrum Medical Industries Inc., Los Angeles, CA.

containing 0.1% sodium azide and packed in a column. The column, whose bed volume was approximately 6 ml (2 mg Protein A/ml gel) was stored and used in a cold room (4°C). The buffer solutions used were 0.14 M sodium phosphate (pH 8.0) or 0.1 M sodium citrate/citric acid (pH 3.0 and pH 6.0) containing azide at 0.05%. Before using the column, it was washed with the pH 3.0 buffer to free bound material and then equilibrated at pH 8.0. The supernatant was applied to the protein A-Sepharose column. The IgG MCA was eluted with the 0.1 M citric acid buffer (pH 6.0). The eluate was monitored at an absorbance of 280 nm[s][t]. The flow rate was 0.5 ml/min and 2.0 ml fractions were collected. Appropriate fractions were pooled and neutralized with Tris buffer, pH 8.8. The antibody titer was determined using a slide agglutination test.

Brush Border Adhesion Test

The procedure described by Sellwood *et al.* (1975) was adapted. With Sellwood's procedure, bacteria and brush borders are incubated together to determine if the bacteria are able to adhere to the brush border fragments. The following test was used to determine if 987P MCA

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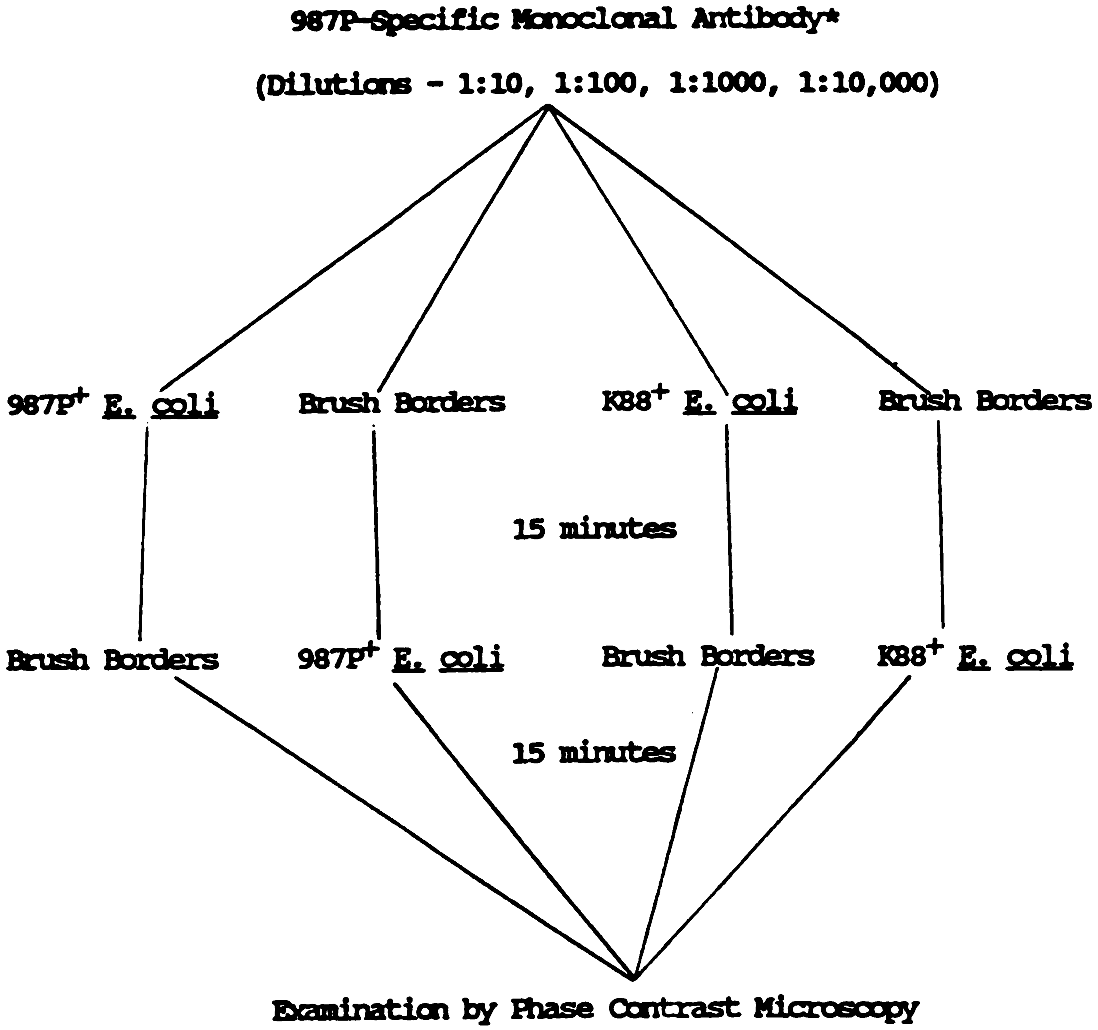
[s] Gilson Absorbance LC Detector, Model III, Gilson Medical Electronics Inc., Middleton, WI.

[t] Gilson Microfractionator, Gilson Medical Electronics Inc., Middleton, WI.

would be able to inhibit the adhesion of 987P-positive *E. coli* to pig intestinal brush borders. The MCA was preincubated either with 987P-positive *E. coli* or with brush border fragments prior to the addition of the third component. The incubation of MCA with brush border fragments before the addition of bacteria mimics a prophylactic situation in the live animal where the MCA would be present in the gut prior to infection.

In the brush border adhesion test, pig intestinal brush borders and 987P-positive *E. coli* were mixed with several different concentrations of 987P MCA. A negative control, using K88-positive *E. coli*, was also used. With each ileal sample, 2 procedures were used. In the first, 0.1 ml of bacteria (987P- or K88-positive *E. coli*) was preincubated for 15 minutes with 0.1 ml of MCA and then mixed with the 0.1 ml of brush borders for 15 additional minutes. In the second procedure, MCA was added to the brush border preparation prior to the addition of the bacteria (Figure 1-1). The stock MCA solution was diluted 1:10, 1:100, 1:1000 or 1:10,000. The titer of the MCA was redetermined prior to each test with a slide agglutination assay. The mixtures were incubated at 37°C with continuous gentle mixing. Each *E. coli* culture was also incubated with 0.1 ml of brush borders alone to determine if adhesion was present in the absence of

Figure 1-1. Brush Border Adhesion Test



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* - titer of stock solution was 10,000

antibody. Additionally, each _E. coli_ culture was incubated with 0.1 ml of KH to determine if autoagglutination was occurring. Also, known 987P- and K88-positive brush border preparations were run as positive controls on each test day. Following incubation, a drop of each of the suspensions was placed on a glass slide and coverslipped. The slide was then viewed by phase contrast microscopy[u] with a 40X objective.

Brush border fragments were indicated as having no adhesion if no bacteria were adherent, partial adhesion if 2 or more bacteria were present and good adhesion if the entire brush border surface contained adherent bacteria.

Statistical Analysis

Data were analyzed using Friedman's test to determine if differences among the eight 987P MCA treatment groups existed for the 987P-positive and K88-positive _E. coli_. The sign test was used to determine differences between paired treatment groups (Steel and Torie, 1980). To perform the sign test and Friedman's test the following values were assigned to the degrees of adhesion: 1 - no adhesion on all fragments, 2 - no adhesion on some fragments and partial adhesion on some fragments, 3 -no adhesion on some fragments and good adhesion on some fragments, 4 - no adhesion on some

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[u] Zeiss Photomicroscope III, Carl Zeiss, Oberkocken, West Germany.

fragments, partial adhesion on some fragments and good adhesion on some fragments, 5 - partial adhesion on all fragments, 6 - partial adhesion on some fragments and good adhesion on some fragments, 7 - good adhesion on all fragments. Differences between groups were considered significant at the $P \leq 0.05$ level.

RESULTS

Brush Border Preparations

The brush border fragments prepared were easily discernible when viewed by phase contrast microscopy. Varying in size, the fragments were semilunar to round in shape with a distinct microvillus border. Characteristically, a halo of bright light was present around each of the brush border fragments. Cell remnants other than the microvillus border also comprised the fragment (Figure 1-2).

Protein Concentrations of the Brush Borders

Five brush border samples were assayed for protein concentrations. The protein concentrations were 1.8 mg/ml, 2.2 mg/ml, 2.3 mg/ml, 2.7 mg/ml and 3.1 mg/ml. The average protein concentration was 2.42 mg/ml.

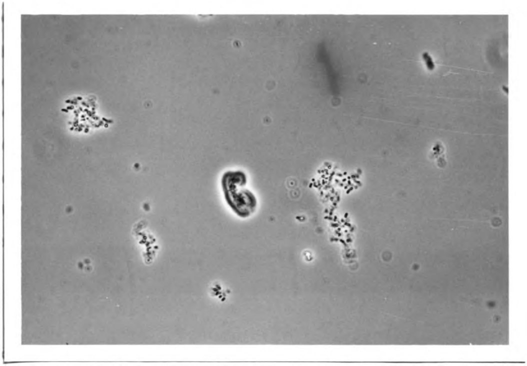


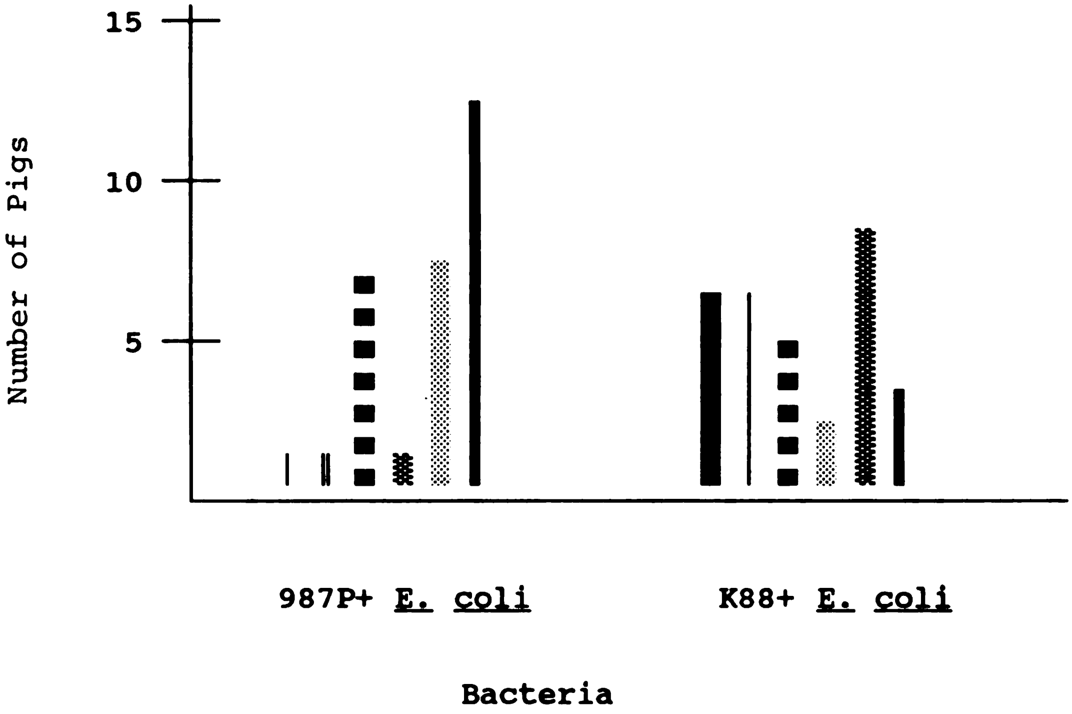
Figure 1-2. Phase contrast photomicrograph of a brush border fragment with no adhesion of 987P-positive E. coli. Notice the moderately sized bacterial aggregates present beside the fragment (480X).

Monoclonal Antibody Production

Using a slide agglutination test, the titer of the antibody in the tissue culture supernatant ranged from 1:100 to 1:1000 depending upon the day of collection. Approximately 350 ml of supernatant were collected per week. A total volume of 29.5 l of tissue culture supernatant was collected. Following ammonium sulfate precipitation, 1 l of supernatant yielded approximately 100 ml of fluid. After affinity chromatography, the 100 ml of supernatant acquired from the ammonium sulfate precipitation step yielded a volume of 8-10 ml with a titer of 10,000. A total volume of 284 ml of 987P MCA were produced with a titer of 10,000.

Adhesion

Prior to incubation with the MCA, ileal samples from each of the 29 pigs all had some degree of adhesion of 987P-positive E. coli to the brush border fragments (Figure 1-3 and Table 1-1). The adhesion ranged from individual brush border fragments with no adhesion to brush border fragments with the entire microvillus surface covered by bacteria (Figures 1-2,4 and 5). Brush border fragments were present in the same preparation with no bacteria adhering to some brush border fragments and varying numbers of bacteria adhering to other fragments. Adhesion for ileal samples incubated with the K88-positive



No adhesion - █

No adhesion on some fragments; partial adhesion on some fragments - |

No adhesion on some fragments; good adhesion on some fragments - ‖

No adhesion on some fragments; partial adhesion on some fragments; good adhesion on some fragment - ▪

Partial adhesion - ▨

Partial adhesion on some fragments; good adhesion on some fragments - ░

Good adhesion - ▌

Figure 1-3. Distribution of the degrees of adhesion of 987P-positive and K88-positive *E. coli* to brush border fragments in ileal samples from 29 pigs.

Table 1-1. The effect of 987P monoclonal antibody on the adhesion of 987P-positive *E. coli* to brush borders (BB) from porcine ileal samples.

Degree of Adhesion	Before Treatment	Treatment*							
		1	2	3	4	5	6	7	8
None	0	29	28	16	3	16	14	15	3
None, Partial	1	0	1	5	6	13	11	5	2
None, Good	1	0	0	0	3	0	1	0	5
None, Partial, Good	7	0	0	2	8	0	2	2	9
Partial	1	0	0	1	1	0	0	0	0
Partial, Good	7	0	0	5	8	0	1	2	3
Good	12	0	0	0	0	0	0	5	7
statistical significance**		b	b	a,h	a,e,f	a,d,g,h	a,d,h	a,e,h	a,c,e,f,g

* Treatments: 1 - (*E. coli* + 1:10 dilution MCA) + brush borders, 2 - (*E. coli* + 1:100 dilution MCA) + brush borders, 3 - (*E. coli* + 1:1000 dilution MCA) + brush borders, 4 - (*E. coli* + 1:10,000 dilution MCA) + brush borders, 5 - (brush borders + 1:10 dilution MCA) + *E. coli*, 6 - (brush borders + 1:100 dilution MCA) + *E. coli*, 7 - (brush borders + 1:1000 dilution MCA) + *E. coli*, 8 - (brush borders + 1:10,000 dilution MCA) + *E. coli*

** ^{a} significantly different from 1 and 2 ($P \leq 0.05$); ^{b} significantly different from 3,4,5,6,7 and 8 ($P \leq 0.05$); ^{c} significantly different from 3 ($P \leq 0.05$); ^{d} significantly different from 4 ($P \leq 0.05$); ^{e} significantly different from 5 ($P \leq 0.05$); ^{f} significantly different from 6 ($P \leq 0.05$); ^{g} significantly different from 7 ($P \leq 0.05$); ^{h} significantly different from 8 ($P \leq 0.05$).

Figure 1-4. Phase contrast photomicrograph of a brush border fragment with good adhesion. Notice the bacteria adhering to the brush border surface (arrow) (480X).

Figure 1-5. Phase contrast photomicrograph of a brush border fragment with partial adhesion. Notice the small aggregates of bacteria present in the background (480X).

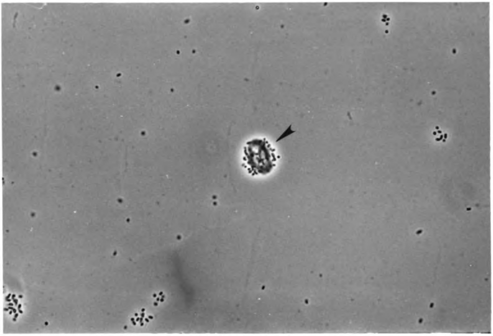


Figure 1-4

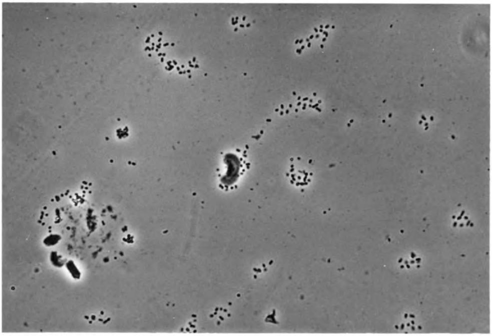


Figure 1-5

Figure 1-6. The effect of preincubation of 987P monoclonal antibody with 987P-positive _E. coli_, prior to the addition of brush borders, on the brush border adhesion test.

No adhesion - █

No adhesion on some fragments; partial adhesion on some fragments - |

No adhesion on some fragments; good adhesion on some fragments - ‖

No adhesion on some fragments; partial adhesion on ■
some fragments; good adhesion on some fragment - ■

Partial adhesion - ▓

Partial adhesion on some fragments; good adhesion on some fragments - ░

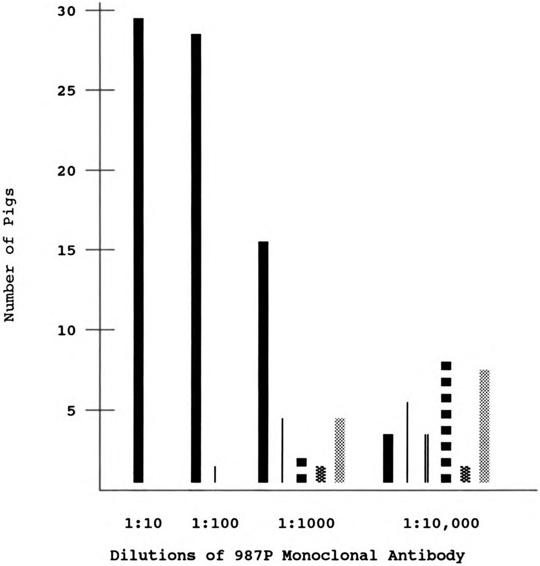

30
25
20
15
10
5
Number of Pigs
1:10
1:100
1:1000
1:10,000
Dilutions of 987P Monoclonal Antibody

Figure 1-6

Figure 1-7. The effect of preincubation of 987P monoclonal antibody with brush borders, prior to the addition of 987P-positive *E. coli*, on the brush border adhesion test.

No adhesion - ▮

No adhesion on some fragments; partial adhesion on some fragments - |

No adhesion on some fragments; good adhesion on some fragments - ‖

No adhesion on some fragments; partial adhesion on some fragments; good adhesion on some fragments - ■ ■

Partial adhesion on some fragments; good adhesion on some fragments - ▒

Good adhesion - ▌

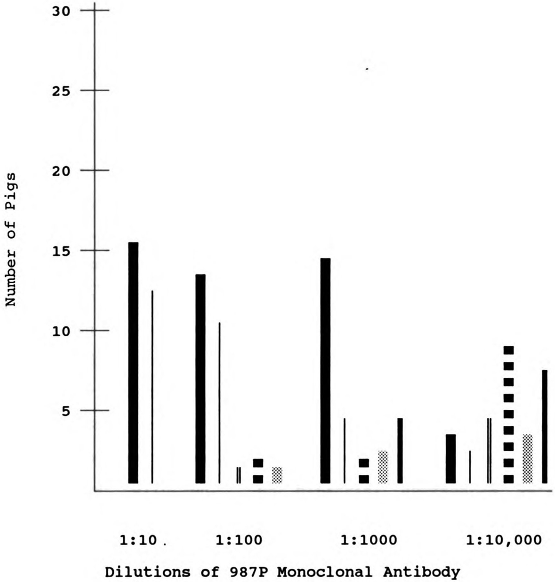

30
25
20
15
10
5
Number of Pigs
1:10
1:100
1:1000
1:10,000
Dilutions of 987P Monoclonal Antibody

Figure 1-7

E. coli ranged from 7 samples with no adhesion to 22 samples with varying degrees of partial and good adhesion (Figure 1-3).

Results of the brush border adhesion test for ileal samples incubated with 987P MCA and 987P-positive *E. coli* are listed in Figures 1-6 and 1-7, and Table 1-1. The 8 different 987P MCA treatment groups showed a statistically significant ($P \leq 0.05$) inhibition of adhesion of 987P-positive *E. coli* to the brush border fragments. There was no statistically significant difference between the extent of adhesion for K88-positive *E. coli* incubated with and without the 987P MCA in the brush border adhesion test.

Brush border preparations containing MCA at the 3 lowest dilutions, 1:10, 1:100 and 1:1000, had large to moderately sized bacterial aggregates present (Figure 1-2). Brush border fragments could not be identified beneath the aggregates. Small aggregates were present at the highest dilution. No bacterial aggregates were present with the K88-positive *E. coli.*

Coadherence occurred with the 987P-positive *E. coli*. The bacteria adhered not only to the brush border membrane, but also concurrently adhered to adjacent bacteria, which in turn may have been adherent to the fragment and/or other bacteria (Figure 1-8).

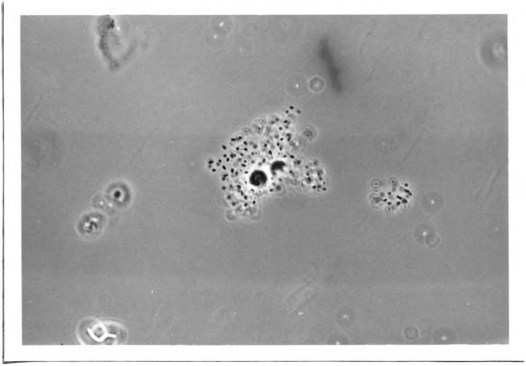


Figure 1-8. Phase contrast photomicrograph of a brush border fragment with good adhesion. Notice the adhesion of bacteria to the brush border surface and to adjacent bacteria (480X).

DISCUSSION

Adhesion of 987P-positive *E. coli* to brush borders was present in all 29 ileal samples tested in the absence of MCA, indicating that all the pigs tested had receptors for the 987P pilus. The results of this study support the statement of Moon *et al.* (1979) who indicated that they had not encountered pigs congenitally resistant to 987P-positive *E. coli*. Adhesion was present in some samples along the entire brush border surface, in both the neonatal pigs and adults, suggesting that age-related resistance to adhesion of 987P-positive E. coli is not the result of a decreased number of receptors, as is the case with K99-positive *E. coli* (Runnels *et al.*, 1980). Also, neonatal and adult pigs had varying degrees of adhesion of 987P-positive and K88-positive *E. coli*. These results are similar to the results of Sellwood (1980a) who described a weak adhesive phenotype where few K88-positive *E. coli* adhered to brush borders *in vitro.*

Inhibition of adhesion of homologous but not heterologous pili was demonstrated by the 987P MCA. With each of the MCA treatments, adhesion to the brush border fragments by 987P-positive *E. coli* was inhibited. No inhibition of adhesion was present when the 987 MCA was either initially mixed with the K88-positive *E. coli* or brush borders and then mixed with the third component, suggesting that the MCA was specific for 987P pili.

Research indicates that pilus antibodies inhibit adhesion by preventing the attachment of piliated *E. coli* to specific receptors. The 987P MCA used in the brush border adhesion test, whether preincubated with the bacteria or brush borders, did appear to prevent 987P-positive *E. coli* from adhering to brush border fragments. Preincubation of the brush borders with the MCA was not as effective as preincubation of the 987P-positive *E. coli* with the MCA. Compared to the other treatment groups, inhibition of adhesion was particularly effective when the 1:10 and 1:100 dilutions of 987P MCA were preincubated with bacteria prior to the addition of brush borders. A 1:1000 dilution of MCA preincubated with 987P-positive *E. coli* was as effective as a 1:10 or 1:100 dilution of MCA preincubated with the brush borders.

The inhibition of adhesion may have been associated with a decreased availability of free bacteria. The bacteria appeared to be prevented from adhering, but large aggregates of bacteria were present in the 1:10, 1:100 and 1:1000 dilutions of MCA. Since the large aggregates were not seen with K88-positive *E. coli*, the aggregates were probably due to the agglutination of 987P-positive bacteria by the 987P MCA. The number of bacteria available to bind to brush border fragments may be severely limited due to the agglutination of bacteria. Kallenius *et al.* (1985) have stated that antiserum or purified Igs, no matter how specific, should not be used as inhibitors of *in vitro* bacterial adhesion since they aggregate bacteria and thus decrease the number of free bacteria. Therefore, observed adhesion values will be decreased in a non-specific manner. It has been suggested that the preferred manner of studying the inhibition of bacterial adhesion by antibodies is to use the antibody in a monovalent form. Intact Igs such as IgG would be cleaved to yield Fab fragments, which are incapable of agglutination. Thus, the inhibition of adhesion by the use of Fab fragments would not be the result of bacterial or fimbrial agglutination but due to specific blocking of the interaction between the epithelial cell receptor and the bacterial pilus (Fachon-Kalweit *et al.*, 1985).

However at the 1:10:000 dilution, the aggregates either were not present or were small, and adhesion was still inhibited to some extent, indicating that inhibition of adhesion can not be attributed solely to bacterial agglutination. Some authors have used subagglutinating titers of antibody in bacterial inhibition adhesion assays to overcome the problem associated with agglutination. Sellwood *et al.* (1975) used a 1:80 dilution of K88 antiserum which inhibited adhesion to brush borders but did not agglutinate at that dilution.

Methods of quantifying the adherent bacteria were based on visual observations alone which may have limited the accuracy of bacterial quantitation. A more specific method of quantifying bacterial adhesion involves the use of radiolabelled bacteria (Hanson *et al.*, 1985; Powell *et al.*, 1976). However, such a procedure generally uses intact epithelial cells rather than brush borders.

Inhibition of *in vitro* attachment of 987P-positive *E. coli* by 987P MCAs suggests that the MCAs should be capable of preventing the adhesion of the 987P pilus to receptors present in the pig intestine *in vivo*. However, responses in an *in vitro* model should not be considered directly indicative of the *in vivo* responses. *In vitro* observations should be evaluated in relation to *in vivo* observations. Bacteria grown in a laboratory don't always express the same surface structures as bacteria grown in animals, and this may also be true of pili. Also, isolated epithelial cells or brush borders don't necessarily retain relevant surface structures when studied *in vitro* (Kallenius *et al.*, 1985).

SUMMARY

A study of 29 pigs was performed to determine if the *in vitro* adhesion of 987P-positive *E. coli* to pig intestinal brush borders could be prevented by 987P MCA. Ileal samples, collected from 17 slaughter-weight pigs and 12 neonatal pigs, were examined using a brush border test. In the brush border adhesion test, pig intestinal brush borders and 987P-positive *E. coli* were mixed with several different concentrations of 987P MCA. Two procedures were used with each ileal sample, either MCA and bacteria or MCA and brush borders were preincubated together prior to the addition of the third component for a total of eight different treatments per sample.

Adhesion of 987P-positive *E. coli* was present in all 29 ileal samples tested in the absence of MCA, indicating that all the pigs tested had receptors for the 987P pilus. Varying degrees of adhesion of 987P-positive *E. coli* were present for the samples. A statistically significant inhibition of adhesion of 987P-positive *E. coli* to brush border fragments by 987P MCA was present whether the 987P MCA was preincubated with the bacteria or brush borders. Preincubation of the brush borders with the MCA was not as effective as preincubation of the 987P-positive *E. coli* with the MCA. Inhibition of adhesion was most effective at the 1:10 and 1:100 dilution of the antibody. Inhibition may have occurred because of prevention of adhesion to

brush border receptors or because of a decreased availability of free bacteria due to agglutination.

In vitro, the 987P MCA appeared to be effective in the inhibition of adhesion of 987P-positive *E. coli* to brush borders.

CHAPTER 2

THE EFFECT OF THE ORAL ADMINISTRATION OF 987P MONOCLONAL ANTIBODY ON THE CLINICAL SIGNS, LESIONS AND ADHESION OF 987P-POSITIVE *ESCHERICHIA COLI* IN GNOTOBIOTIC PIGS

CHAPTER 2

THE EFFECT OF THE ORAL ADMINISTRATION OF 987P MONOCLONAL ANTIBODY ON THE CLINICAL SIGNS, LESIONS AND ADHESION OF 987P-POSITIVE *ESCHERICHIA COLI* IN GNOTOBIOTIC PIGS

INTRODUCTION

Immunologically, the newborn pig is relatively defenseless and thus highly susceptible to infection. Correlation of the 3 ages of peak incidence of porcine enteric colibacillosis - neonatal, 3 weeks and immediately postweaning - with periods of antibody deficiency indicate that such a deficiency predisposes pigs to ETEC (Moon, 1974). Since adhesion of ETEC to the intestine is an essential prerequisite for the development of enteric colibacillosis, antibody-mediated prevention of bacterial adhesion is a potential defense mechanism against *E. coli*. Passive immunization, via the absorption of antibodies from colostrum and milk, of the neonatal pig prior to its exposure to an infectious agent protects the pig from infection.

Vaccination of sows prior to farrowing helps to induce protective levels of antibodies in the sow's colostrum and milk, thus providing passive immunity to the newborn pig. Currently, several vaccines have been developed for the protection of pigs against neonatal enteric colibacillosis. Research has demonstrated that protection correlates with antipilus antibodies in the colostrum and milk (Acres *et al.*, 1979; Nagy *et al.*, 1978; Rutter *et al.*, 1976). Several researchers believe that colostral antibodies prevent the colonization of ETEC by blocking adhesion (Nagy *et al.*, 1978; Rutter, 1975; Rutter *et al.*, 1973, 1976).

Several early reports in the literature have demonstrated a protective effect of orally or parenterally administered hyperimmune serum, colostrum and milk against experimental challenge with ETEC in gnotobiotic and conventional pigs (Kohler, 1967, 1974; Kohler and Bohl, 1966; Miler *et al.*, 1975; Miniats *et al.*, 1970; Rejnek *et al.*, 1968; Smith, 1972; Smith and Linggood, 1971b; Svendsen and Wilson, 1971; Wilson and Svendsen, 1971). Recently, an alternative method for the direct passive immunization of newborn animals against ETEC, the oral administration of *E. coli* pilus-specific MCA, has been developed. Knowledge concerning the effectiveness of pilus-specific MCAs against ETEC in neonatal animals is limited.

One of the first reports involved the oral administration of *E. coli* K99 MCA to calves (Sherman *et al.*, 1983). In that study, there was a statistically significant reduction in the mortality rate and the severity and duration of diarrhea in the treated calves versus the control calves. However, there was no difference in the incidence of diarrhea. Several explanations were postulated for the diarrhea which occurred in animals which received the K99 MCAs. It was speculated that the amount of MCA used may not have completely blocked colonization. Also, it was suggested that the number of bacteria in the challenge inoculum might have been large enough to produce sufficient enterotoxin without adhesion to the mucosa to cause diarrhea. The previous study led to the development of a commercially available K99 MCA for calves, Genecol 99 (Molecular Genetics, Inc. Minnetonka, MN).

Studies with pigs using K88- (Foged et al., 1986; Sadowski, 1984), K99- (Mainil et al., 1987; Sadowski et al., 1983) and 987P (Sadowski et al., 1986) MCAs have also been performed. Few animals were used in the studies which were primarily clinical in nature. The effectiveness of the antibody varied from protection against challenge, based on an increased survival rate and a delayed onset of diarrhea, to no prophylactic effect. Interestingly, both treated and control animals developed diarrhea in the majority of the studies.

In this study, MCAs specific for the 987P pilus were administered to gnotobiotic pigs in an attempt to protect the pigs from diarrhea due to 987P-positive *E. coli*.

The objectives of this study were: 1) To determine if the clinical signs and lesions produced by 987P-positive *E. coli* in gnotobiotic pigs could be altered by the oral administration of *E. coli* 987P MCAs and 2) To determine if the *in vivo* adhesion of 987-positive *E. coli* to the intestinal mucosa of gnotobiotic pigs could be prevented by the oral administration of *E. coli* 987P MCA.

MATERIALS AND METHODS

E. coli Strains

The 987P-positive strain of *E. coli* used to infect the gnotobiotic pigs was strain 81-1469. The culture, obtained from Dr. David Francis, Department of Veterinary Science, South Dakota State University, Brookings, SD, was incubated[a] aerobically at 37°C for 8 hours in trypticase soy broth[b] prior to challenge. Following incubation, the culture was centrifuged at 1500 g for 20 minutes. The supernatant was discarded, and the bacteria were washed in phosphate buffered saline and centrifuged 1 more time. The pellet was resuspended to an optical density between

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[a] Thelco Incubator, Model 4, Precision Scientific, Chicago, IL.

[b] Baltimore Biological Laboratories, Cockeysville, MD.

.075 and .085 at 560 nm[c]. The suspension contained approximately 10^8 colony-forming units per ml. Bacteria were checked for piliation using a standard slide agglutination test with 987P antiserum (refer to Chapter 1). The challenge inoculum was aseptically placed in 5 to 10 ml sterile glass ampules. The ampules were heat sealed with a propane torch and aseptically transferred into the isolators just prior to challenge of the pigs.

Quantitation of E. coli in Inocula

One milliliter of the bacterial culture was pipetted into 9 ml of peptone water and ten 10-fold dilutions were made in peptone water. Using a standard pour plate technique, 1 ml of each dilution was plated on MacConkey agar in duplicate and then incubated for 22 hours at 37°C. Colony-forming-units were counted using a digital colony counter, and the average colony count for each dilution was determined. Duplicate dilutions were done for each bacterial culture.

Monoclonal Antibody

The production of 987P MCA was the same as that previously described in Chapter 1. The MCA and PBS placebo were placed in 5 to 10 ml sterile glass ampules and heat sealed. The ampules were then aseptically

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[c] Shimadzu UV Visible Recording Spectrophotometer, Model UV-260, Shimadzu Coporation, Kyoto, Japan.

transferred along with the challenge inocula into the appropriate isolators.

Experimental Animals

Eight litters of gnotobiotic pigs were used (Figure 2-1). Pregnant sows were obtained from Michigan State University's Swine Center.

Technique for Obtaining Gnotobiotic Pigs

The gnotobiotic pigs were obtained using a technique described by Waxler et al. (1966). All flexible, transparent, vinyl film isolators used in obtaining and rearing the gnotobiotic pigs were sterilized prior to use. Cages were sterilized in an autoclave at 121°C for 30 minutes prior to passage into detergent-cleaned isolators. The isolators and the cages were subsequently sprayed with an aerosol of 2% peracetic acid[d]. The air entering the sterile isolators passed through sterile air filter units. Feeding and other equipment were sterilized in an autoclave at 121°C for 30 minutes in a stainless steel, filter-equipped cylinder before being passed into the isolators via a transfer sleeve sterilized with peracetic acid. Cans of milk were also sprayed with peracetic acid prior to passage into the isolator.

The pregnant sow was anesthetized on the 112th day of gestation. Using an 18 gauge, 6 inch needle, an

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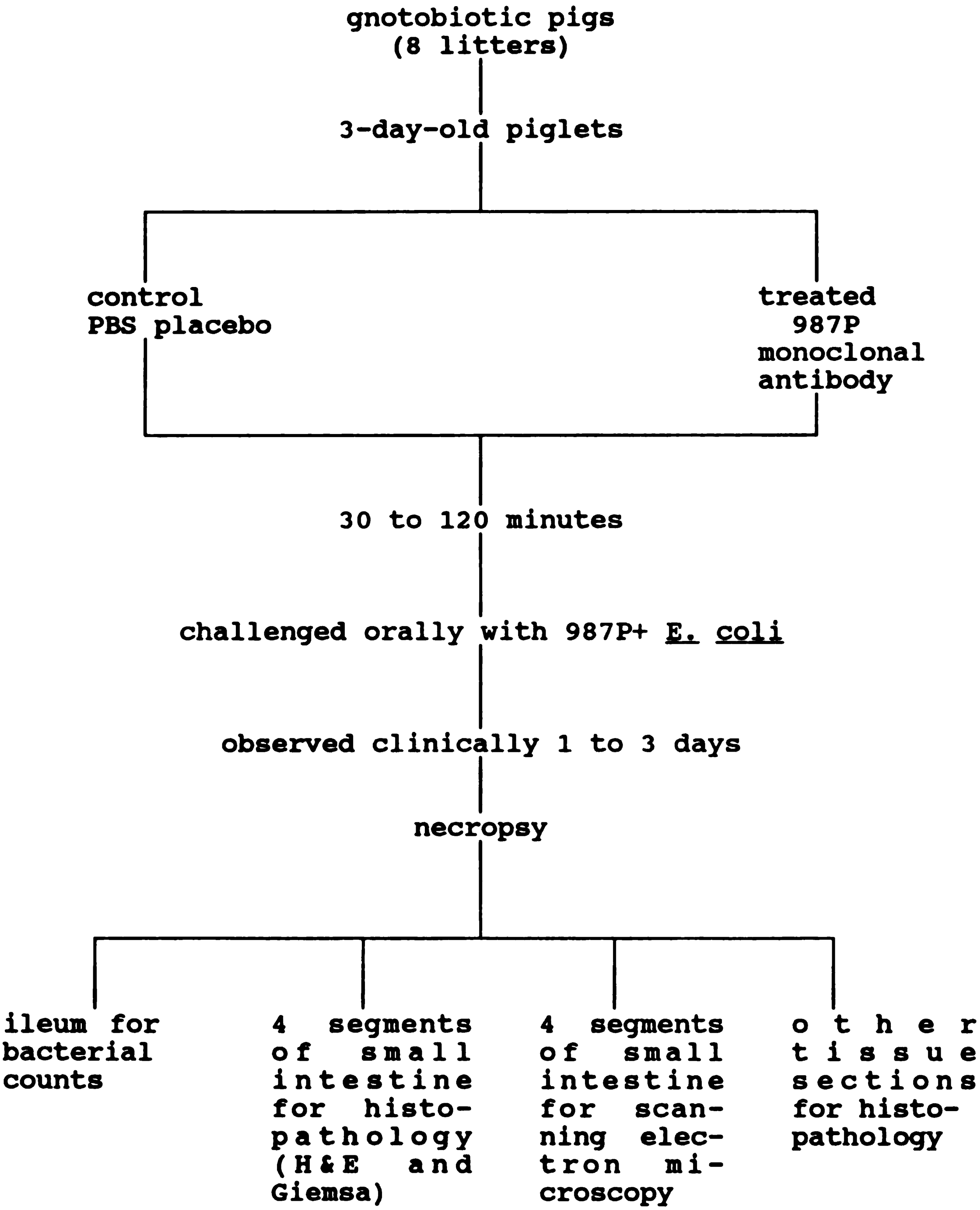
[d] FMC Corporation, New York, NY.

Figure 2-1. Experimental Design



epidural was performed by injecting 15 ml of lidocaine[e] at the lumbosacral articulation (Getty, 1963). Two to 3 ml of a tranquilizer[f] were then intramuscularly injected into the sow. Next, the sow was secured on the surgical table in right lateral recumbency. The left flank was then clipped, shaved, and surgically prepared with Betadine Surgical Scrub[g], Betadine Solution[f] and 70 % ethyl alcohol. Chloroform was then applied to the left flank and dried with sterile towels. Next, the flank's surgical site and the vinyl film covering the 12-inch port in the floor of the surgical isolator were sprayed with a surgical adhesive[h]. The 12-inch port was then positioned over the surgical site and the port was secured in place with 3 cords.

The surgeon, operating through shoulder-length rubber gloves, then cut through the vinyl floor of the 12-inch port and the sow's skin with a cautery unit[i]. The incision was approximately 9 inches in length and extended from the most dorsal aspect of the port to near the most ventral aspect. As the incision was made, the cut edges

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[e] Lid-O-Cain 2%, Butler Co., Columbus, OH.

[f] PromAce (Acepromazine maleate), Fort Dodge Laboratories Inc., Fort Dodge, IA.

[g] Purdue Frederick Co., Norwalk, CT.

[h] Vi-Drape, Parke-Davis, Detroit, MI.

[i] National Electric Instrument Co., Inc., Long Island, NY.

of the vinyl film and the abdominal skin were clamped together with Allis tissue forceps, and the forcep handles were affixed to rubber bands extending through holes in the border of the 12-inch port. The remaining layers of the abdominal wall were then incised with scissors. Each pig was removed from the uterus by a separate incision in the uterine wall. Umbilical clamps[j] were placed on the umbilical cord of each pig prior to cutting the cord. The pig was then aseptically transferred into an attached transport isolator where it was dried and placed in a cage. When all the pigs had been removed, the sow was euthanatized by an intravenous injection of sodium pentobarbital[k].

After delivery of the pigs, the transport isolator was disconnected from the sleeve attaching it to the surgical isolator. Pigs were then randomly placed in the rearing isolators and housed in individual cages within the isolators with 4 cages per isolator. Control animals were housed in separate isolators. The room temperature was 30-32°C. Equipment for feeding and for microbiologic determination was present in each isolator. The diet

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[j] Double Grip Disposable Cord-Clamp, Hollister, Inc., Chicago, IL.

[k] Butler Company, Columbus, OH.

consisted of evaporated milk[l] and each pig was fed ad lib 3 times a day.

Bacteriologic Monitoring of Isolators

Swabs were taken from each isolator just prior to the time the pigs were exposed to 987P-positive E. coli and subsequent to the termination of the experiment. Swab specimens were obtained from rectal contents, waste material from the cages, contents of feeding trays and the plastic isolator. Material from the swabs was suspended into 0.5 ml of Brain Heart Infusion (BHI) broth[b], and 0.1 ml of this suspension was inoculated into enriched blood agar (BHI with 5% defibrinated sheep blood, 5% heat-inactivated horse serum and 1% yeast extract powder) and MacConkey agar[m]. The cultures were then aerobically incubated at 27°C, 37°C and 56°C. The CDC anaerobic blood agar was also inoculated and anaerobically incubated at 27°C, 37°C and 56°C in a Bio-Bag environmental chamber type A[n]. The BHI suspension was also inoculated into thioglycollate medium[m] supplemented with Hemin and vitamin K and incubated aerobically in tightly screwed capped tubes at 27°C, 37°C and 56°C. The primary plating medium

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[l] Carnation Company, Los Angeles, CA.

[m] Difco Laboratories, Detroit, MI.

[n] Marion Laboratories, Kansas City, MO.

and thioglycollate medium were checked daily for 2 weeks. Isolated organisms were examined and identified.

Treatment of Pigs

In the initial trial, 3 litters of pigs were used to determine the optimum amount of MCA to be administered to the pigs. The study was a paired study, for each control animal there was a treated animal. Control and treated animals were randomly assigned. Three-day-old pigs were fasted 18 hours prior to the administration of the 987P MCA. In the first litter of pigs, the MCA was administered in the backs of the piglets' mouths with a syringe. In the second litter of pigs the MCA was given via a stomach tube, and in the last 6 litters of pigs, the MCA was administered via a bottle. The MCA was mixed with 5-10 ml of milk in the bottle, and the pigs were then fed milk immediately after the administration of the MCA. Control pigs were given a phosphate buffered saline placebo. One half hour to 2 hours later the pigs were challenged with 1 ml of the bacterial culture (Table 2-1).

Determination of Clinical Signs

Pigs were observed 3 times daily, at each feeding, for clinical signs of anorexia, vomiting, dehydration, depression and diarrhea.

Table 2-1. Treatment protocol for the 8 litters of gnotobiotic pigs.

Litter Number	No. Pigs[a]	Age of Pigs	MCA Treatment[b]	*E. coli* Challenge[c]	Method of Inoculation	Time of Challenge[d]	Observation Period
1	10	3 days	0.5 ml (2) 1.0 ml (3)	10^9	mouth	2 hours	3 days
2	5	3 days	3.0 ml	10^8	stomach tube	0.5 hours	1 day
3	11	5 days	4.0 ml (3) 7.0 ml (3)	10^{10}	bottle	0.5 hours	3 days
4	12	4 days	10.0 ml	10^8	bottle	0.5 hours	2 days
5	14	4 days	10.0 ml	10^8	bottle	0.5 hours	2 days
6	5	3 days	12.0 ml	10^8	bottle	0.5 hours	2 days
7	11	2 days	7.0 ml	10^8	bottle	0.5 hours	2 days
8	10	3 days	10.0 ml	10^8	bottle	0.5 hours	2 days

[a] Control and treated pigs were equally divided except for litter 2 (2 control pigs), litter 3 (5 control pigs), litter 6 (2 control pigs) and litter 7 (3 control pigs).

[b] 987P monoclonal antibody (MCA) with a titer of 10,000/ml determined by a slide agglutination test.

[c] Number of organisms per milliliter.

[d] Time after MCA administration.

Necropsy Procedure

Twenty-four to 72 hours (Table 2-1) after exposure to 987P-positive *E. coli* the pigs were removed from the isolator and euthanatized by an overdose of sodium pentobarbital administered intravenously. A complete gross examination was performed. The small intestine was removed, and 4 equally spaced segments were fixed in 10% buffered formalin for histologic examination. Four additional segments of small intestine were taken for scanning electron microscopic evaluation from half the control and treated pigs in the litter. A 10 cm segment of ileum was taken from each pig for microbiologic culture. In addition to the small intestinal sections, sections of lung, liver, kidney, spleen, lymph node, stomach, cecum, spiral colon, pancreas and adrenal were also formalin-fixed.

Preparation of Histologic Sections

The formalin-fixed sections were routinely processed, embedded in paraffin, cut by a microtome into 6 μm sections and stained with hematoxylin and eosin, and Giemsa stains. Giemsa stained sections were evaluated to determine the relative numbers of bacteria adherent to the mucosa based on a modified association index (Bertschinger *et al.*, 1972). Two criteria were used. The first criterion was based on the tendency for bacteria to be contiguous to epithelial cells and was graded as 1 for none to 5 for maximum. The second criterion was based on the number of bacteria adherent to the villi and was graded as 1 for none to 5 for maximum. The adhesion index for the histologic section was then determined by multiplying the values for the 2 criteria.

Preparation of Scanning Electron Microscopic Sections

One centimeter sections of the small intestine were opened along their mesenteric border and washed with cold 0.1 M PBS until the intestinal contents were removed. The intestinal sections were then fixed individually in cold 2% glutaraldehyde for 8 hours. The fixative was replaced, and fixation was continued for 48 hours at 4°C. The small intestinal segments were then washed 3 times in cold 0.1 M PBS and once with distilled water, trimmed to 3 mm, and postfixed in 1% osmium tetroxide[o] in 0.1 M cacodylate buffer[p] (pH 7.2) at 4°C overnight (Hadad _et_ _al._, 1982a). The tissues were then washed 6 times in distilled water during a period of 10 to 15 minutes and fixed by a modified thiocarbohydrazide (TCH) method (Malick and Wilson, 1975). A 1% saturated solution of TCH[o] in distilled water was freshly prepared and filtered prior to its use. The tissues were incubated in the TCH solution for 20 to 30 minutes at room temperature with gentle

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[o] Polysciences, Inc., Warrington, PA.

[p] Electron Microscopy Sciences, Fort Washington, PA.

agitation. The tissues were then rinsed with 6 changes of distilled water during a period of 10 to 15 minutes. Next, the tissues were placed in a 1% osmium tetroxide solution prepared with distilled water for 2 to 3 hours at room temperature with gentle agitation. The tissues were rinsed 6 times with distilled water. A fresh TCH solution was prepared, and the tissues were incubated in the solution for 20 to 30 minutes. The tissues were rinsed 6 times with distilled water. The osmium tetroxide step was repeated and the tissues were rinsed 6 more times. A standard dehydration in alcohol was performed. The tissues were then critical point dried[q] and mounted on aluminum stubs. Samples were examined using a scanning electron microscope[r].

Microbiologic Quantitation of E. coli

To further determine the extent of colonization, a 10 cm segment of ileum was removed from each pig. The luminal contents of each segment were washed out with 10 ml of a 0.3% solution of peptone water[l]. The volume of each luminal washing and each intestinal wall sampling were adjusted to 30 ml with peptone water. The samples were then individually homogenized at high speed for 1 minute using a Sorvall Omni-Mixer[q]. One milliliter of the

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[q] Ivan Sorvall, Inc., Newton, CT.

[r] JEOL JSM-35C Scanning Electron Microscope, Japanese Electron Optics Laboratory, Tokyo, Japan.

resultant mixture was then pipetted into 9 ml of peptone water, and eight 10-fold serial dilutions were made in peptone water. Duplicate dilutions were done for each sample. Using a standard pour plate technique, 1 ml of each dilution was plated on MacConkey agar in duplicate and then incubated for 22 hours at 37°C. Colony-forming-units were counted using a digital colony counter[s], and the average colony count for each dilution was determined. The formula used to calculate the number of lactose-fermenting bacteria was the following:

$$\text{Con} = 1/10^{-a} \times 30 \times b.$$

The original concentration of the numbers of lactose-fermenting bacteria per 10 cm of ileum corresponds to Con, the dilution from which the inoculum was taken corresponds to 10^{-a}, the total volume of peptone water into which the 10 cm segment was homogenized corresponds to 30, and the average number of colonies counted for that dilution corresponds to b (Nagy _et al._, 1976).

MCA Activity in Gastric and Cecal Contents

Six 3-day-old pigs, which had been removed from the sow shortly after suckling colostrum, were used. Three pigs were bottle fed 10 ml of 987P MCA mixed with 2 oz of milk. The 3 control pigs were fed 10 ml of PBS mixed with milk. The piglets were euthanatized 2 hours later.

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[s] Lab-Line Digimatic Colony Counter, Lab-Line, Inc., Melrose Park, IL.

Gastric contents were collected and centrifuged. The supernatants were diluted 1:10, 1:100, 1:1000 and 1:10,000 and used in a slide agglutination test (refer to Chapter 1) with 987P-positive and K88-positive *E. coli*.

Three 3-day-old pigs were also removed from another sow shortly after suckling colostrum. Two of the pigs were treated with MCA as described previously and the third pig received PBS. The piglets were euthanatized 6 hours later. The stomach and cecal contents were collected and centrifuged. The slide agglutination test was performed as described previously. Also, a brush border adhesion test (refer to Chapter 1) was performed with preincubation of the 4 dilutions of gastric and cecal supernatants with 987P-positive and K88-positive *E. coli* prior to the addition of brush borders.

Statistical Analysis

Data were analyzed using a Chi-square test to determine if differences in morbidity, mortality and colonization existed between the control and treated animals in each litter. A paired t test was used to determine differences between bacteriologic counts for the control and treated pigs in each litter. Bacteriologic count data were transformed using a square root transformation.

Differences between groups were considered significant at the $P \leq 0.05$ level (Steel and Torie, 1980).

RESULTS

Bacteriologic Monitoring

Prior to challenge, no bacteria were detected in any of the samples collected from the isolators housing the 8 litters. At the time of euthanasia, samples from all of the isolators except 1 yielded pure cultures of 987P-positive *E. coli*. No bacteria were isolated from one isolator in litter 7.

Clinical Signs

Before challenge with 987P-positive *E. coli*, the gnotobiotic pigs were active, alert and healthy. Usually within 2 days the pigs were nursing well from bottles. Their yellowish to tan feces were firm to pasty in texture.

Within 5 to 16 hours of challenge (Table 2-2), the pigs began to show signs typical of enteric colibacillosis. Clinical signs between the treated and control animals were very similar, particularly in litters 4-8. Clinical signs in the 8 litters of pigs consisted of diarrhea, dehydration, anorexia, depression and weakness. All of the infected pigs, both control and treated, had diarrhea. The pale to golden yellow feces ranged from watery to pasty in consistency. Usually during the day,

Table 2-2. Onset of clinical signs, morbidity, mortality and intestinal colonization in gnotobiotic pigs given 987P monoclonal antibody and exposed to 987P-positive *E. coli*.

Litter Number	Onset of Clinical Signs (hours)		Morbidity (%)		Mortality (%)		Number of Animals Colonized[a]	
	C[b]	T	C	T	C	T	C	T
1	16	16	100	100	20	0	2	0
2	8	8	100	100	0	0	0	0
3[c]	5	5 (2-4ml), 10 (1-4ml,2-7ml), 12 (1-7ml)	100	100	60	67 (4 ml) 33 (7 ml)	5	3 (4 ml) 2 (7 ml)
							0	1
4	11	11	100	100	0	0	0	0
5	12	12	100	100	0	0	0	0
6	11	11	100	100	0	0	1	1
7	14	14	60	100	0	17	0	0
8	10	10	100	100	0	0	0	0

[a] Colonization determined by microbiologic bacterial counts.

[b] C = Control; T = Treated.

[c] Two of the control pigs and 2 of the 4 ml treated pigs were euthanatized.

while the pigs were being fed, diarrhea was present. However, little evidence of diarrhea was present the next morning. The diarrhea would ensue after the morning feeding. Generally, the piglets' appetites were good, but occasionally the piglets became anorectic, particularly those piglets which had severe diarrhea or were near death. At the time of euthanasia, the diarrhea had usually abated and the pigs were recovering from the infection

In litter 1, initially all the pigs had similar clinical signs, but by the end of the 3 day observation period 1 of the control pigs had died and another control pig was extremely depressed, thin, anorectic and dehydrated in comparison to the other pigs. At the time of euthanasia the 8 other pigs appeared to be recovering from the infection. Sixteen hours after infection, the animals began to show signs of mild diarrhea characterized by a yellow, pasty stool which stained the piglets' hindquarters. However, the piglets remained alert and active and had good appetites. Similar clinical signs were observed the next day. One control animal was depressed though. The next morning, 44 hours after challenge, the animals' stools were firmer. One control pig was still depressed and anorectic. Four hours later, mild diarrhea was again present in all the pigs, and the control animal was dead.

Animals in litter 2 were observed clinically for 24 hours. Only mild diarrhea was observed in the piglets.

In litter 3, within 5 hours, all of the pigs except for the pigs treated with 7 ml of MCA and 1 of the pigs treated with 4 ml of MCA had damp hindquarters. Also, 3 of the control pigs were depressed. After 10 hours all except 1 of the pigs treated with 7 ml of MCA had diarrhea. By the next morning 3 of the control pigs had died. The other 2 control pigs were weak, anorectic, depressed and dehydrated so they were euthanatized and necropsied. On the following day, 2 of 3 pigs in the group treated with 4 ml of MCA not only had diarrhea, but were depressed, anorectic, weak and 5-10% dehydrated so they were euthanatized and necropsied. On the third and final day after challenge, 1 of the pigs treated with 7 ml of MCA had died. The 3 remaining MCA treated pigs were thin and stained with feces, but the 2 pigs treated with 7 ml of MCA were also alert, active and hungry.

In litters 4-8, the clinical signs were similar to those observed previously but not as severe. Signs for the treated and control animals were very similar. In litter 4, the control pigs appeared to have slightly worse diarrhea and were anorectic near the end of the observation period. Initially, the diarrhea in the control animals from litter 6 was worse than the diarrhea in the treated animals. Later, the clinical signs were the same in the treated and control groups. Two control pigs in litter 7 remained healthy throughout the observation period. One of the treated pigs in litter 7, the runt of the litter, died the day after it was infected. Otherwise clinical signs in the 2 groups in litter 7 were very similar.

Gross Lesions

Minimal gross lesions were observed. In the 8 litters examined, the lesions differed very little between the control and treated animals. Gastrointestinal lesions consisted of variable amounts of fluid in the small intestine, cecum and colon, occasional distention of the bowel, and soft to liquid feces in the rectum. Stomachs were often distended with milk. Dehydration, urate crystals in the kidney, inflamed vulvas, anteroventral pneumonia and occasional gastric fundic hemorrhage were also present in some of the piglets.

Few gross lesions were present in litter 1. Liquid contents were present in the colon of all the pigs. The control pig which died from the infection and 1 other control pig were gaunt and dehydrated.

The small intestines and spiral colons of both control and treated animals were distended with fluid in litter 2. Anteroventral bronchopneumonia was also present in the piglets.

Grossly, in control and treated pigs in litter 3, the small intestines, primarily the distal half of the jejunum and the ileum, were distended with fluid and were hyperemic. Gastric fundic hemorrhage was present along the greater curvature of the stomachs of approximately half the control and treated animals. Gastric lesions and small intestinal hyperemia were present in animals which died from the infection or in animals which were euthanatized because of severe illness. Petechial hemorrhages were present on the surface of the kidney of 1 treated pig. Aspiration pneumonia was present in several of the pigs. The piglets' stomachs also tended to be distended with milk, and liquid contents were present in their colons. At the end of the observation period, 1 of the pigs treated with 7 ml of MCA had grossly normal intestines, and the stool was beginning to form.

Gross lesions in litters 4-8 were similar and consisted of variable amounts of fluid in the colon, soft pasty to liquid stools, milk distended stomachs, aspiration pneumonia and inflamed perineums.

Histopathologic Lesions

Intestinal lesions, when present, were generally confined to the distal half of the small intestines, primarily the ileum and lower jejunum. Mild to moderate neutrophilic infiltration was present in the lamina propria of some small intestinal sections. Post mortem autolysis in some of the pigs which died from the infection made the interpretation of intestinal lesions difficult.

The degree of adhesion of 987P-positive E. coli to the intestinal mucosa varied but was generally low (Table 2-3). A few piglets, primarily animals from litter 3, had numerous bacteria adherent to the microvillous surface of intestinal villi extending from the crypts to the tips of the villi (Figures 2-2 and 2-3). In some pigs only a small number of adherent, randomly scattered bacteria were present in the small intestine (Figure 2-4). It was not uncommon to find one section of the small intestine with adherent bacteria and an area adjacent to the section with no adherent bacteria (Figure 2-2). In most of the piglets, particularly litters 4-8, no adherent bacteria whatsoever were present throughout the small intestine. Often in areas where no adherent bacteria were present, large numbers of bacteria were seen adjacent to the small intestinal villi entrapped in mucus but not contiguous to the brush border surface (Figure 2-5). Various numbers of bacteria were present in the lumen of most intestinal sections.

In litters 2-8, the majority of the piglets had mild to moderate, multifocal, neutrophilic bronchopneumonia consisting of neutrophils in bronchioles and surrounding alveoli. In litter 3, piglets with gross lesions of

Table 2-3. Adhesion index for 987P-positive *E. coli* to gnotobiotic pig small intestinal sections.

Intestinal Section[a]		Litter Number															
		1		2		3		4		5		6		7		8	
		M[b]	R	M	R	M	R	M	R	M	R	M	R	M	R	M	R
1	C	1	-	1	-	1	-	1	-	1	-	1	-	1	-	1	-
	T	1	-	1	-	1	-	1	-	1	-	1	-	1	-	1	-
2	C	3.25	1-9	1	-	4.25	2-12	1.2	1-2	1	-	1	-	1	-	1	-
	T	1.6	1-2	1	-	1	-	1	-	1	-	1	-	1	-	1	-
3	C	5.25	1-16	1	-	7.5	1-25	1.16	1-2	1	-	1	-	1	-	1	-
	T	2.2	1-4	1	-	1.75	1-3	1.16	1-8	1	-	1	-	1	-	1	-
4	C	2.75	1-4	1	-	11.2	1-20	1.16	1-2	1	-	1	-	1	-	1	-
	T	2.8	1-4	1	-	9.5	1-12	2.16	1-8	1	-	1	-	1	-	1	-

[a] 1 = Duodenum; 2 = Upper Jejunum; 3 = Distal Jejunum; 4 = Ileum; C = Control; T = Treated. Post mortem changes in 1 control animal in litters 1 and 3 and 2 treated animals in litter 3. Intestinal sections from these animals were not included in the adhesion index.

[b] M = Mean; R = Range.

Figure 2-2. Photomicrograph of a section of ileum with bacteria adherent to the small intestinal villi extending from the crypts to the tips of the villi (arrows). Notice the absence of adherent bacteria to the adjacent small intestinal villi (Giemsa stain, 120X).

Figure 2-3. Photomicrograph of a section of ileum with numerous rod-shaped bacteria (arrows) adherent to the lateral surfaces of the intestinal villi (Giemsa stain, 480X).

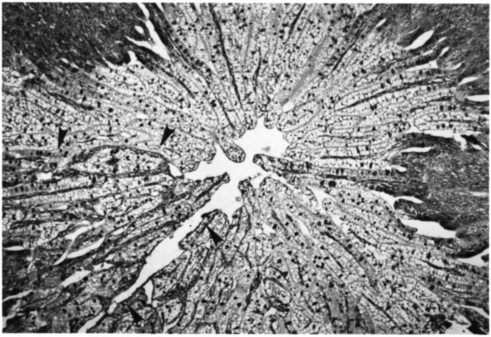

Figure 2-2

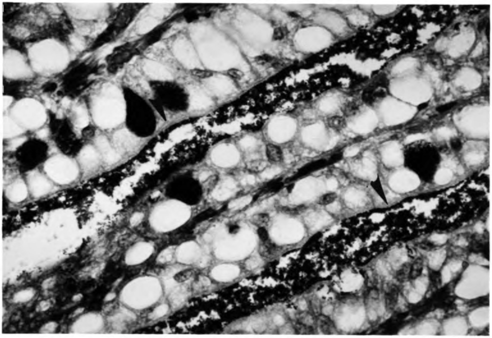

Figure 2-3

Figure 2-4. Photomicrograph of a section of ileum with few bacteria adherent to the lateral surfaces of the villi (arrows) (Giemsa stain, 480X).

Figure 2-5. Photomicrograph of a section of ileum with bacteria entrapped in mucus (arrow) but not contiguous to the brush border surface (Giemsa stain, 480X).

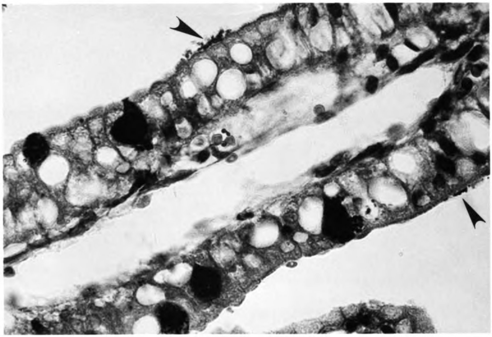

Figure 2-4

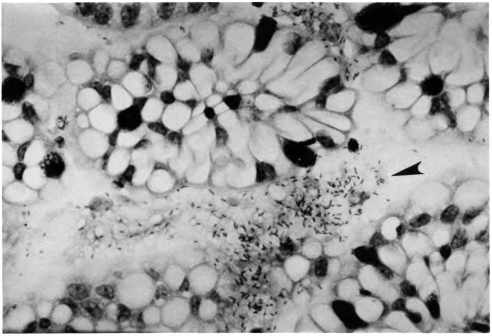

Figure 2-5

gastric fundic hemorrhage had hyperemic blood vessels present from the tunica muscularis to the mucosa of their stomachs. A few vessels appeared to contain fibrinous thrombi. Lesions were difficult to detect in many of the stomachs because of post-mortem changes. Moderate infiltrations of neutrophils were present from the serosal to the mucosal surface of the stomachs. Also, sections of small intestine contained numerous hyperemic blood vessels in the mucosa with occasional thrombi. Generally, histopathologic lesions were not found in any of the other organs examined.

Scanning Electron Microscopic Lesions

In most of the intestinal samples examined via scanning electron microscopy no visible bacteria were attached to the small intestinal villi. In the segments of distal jejunum and ileum of 2 animals in litter 3, 1 control and 1 treated, bacteria were seen attached by their sides and poles to intestinal epithelial cells, primarily on the sides of the villi (Figures 2-6 and 2-7).

Microbiologic Quantitation

Bacterial counts for intestinal contents and the intestinal wall are listed in Tables 2-4 - 2-7. Generally, the numbers of viable E. coli in the lumen and intestinal wall were low, less than 10^8, except for litter 3. Numbers ranged from 0 to $>10^{12}$ E. coli.

Figure 2-6. Scanning electron micrograph of section of ileum with bacteria adherent to the lateral surface of the intestinal villi (arrow) (320X).

Figure 2-7. Scanning electron micrograph of section of ileum with bacteria adherent to the brush border surface of the intestinal epithelium (5,300X).

Figure 2-6

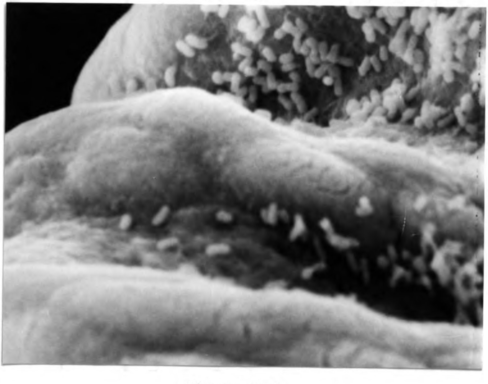

Figure 2-7

Table 2-4. Numbers of viable E. coli in the luminal contents of 10 cm segments from pigs in litters 1-4.

LITTER NUMBER							
1		2		3[a]		4	
C[b]	T	C	T	C	T	C	T
5.2×10^4	6.5×10^5	$<10^5$	$<10^4$	1.2×10^8	4.3×10^9[c]	2.4×10^5	1.2×10^6
1.5×10^5	6.7×10^6	$<10^2$	5.5×10^4	6.7×10^6	1.9×10^9[c]	5.0×10^4	$<10^3$
6.4×10^9	1.8×10^6		$<10^2$	1.1×10^{12}	7.7×10^8[c]	$<10^5$	2.9×10^6
1.1×10^6	4.5×10^2			2.1×10^{11}	6.4×10^6[d]	$<10^7$	8.7×10^6
1.6×10^6	7.5×10^5			$>10^{11}$	3.2×10^6[d]	7.8×10^4	1.7×10^5
					2.7×10^9[d]	$<10^5$	2.1×10^4

[a] Statistically significant difference between treated and control animals ($P \leq 0.05$).

[b] C = Control; T = Treated; paired animals are listed side by side.

[c] Pig treated with 4 ml of 987P monoclonal antibody.

[d] Pig treated with 7 ml of 987P monoclonal antibody.

Table 2-5. Numbers of viable *E. coli* associated with the intestinal wall in 10 cm segments of ileum from pigs in litters 1-4.

LITTER NUMBER							
1		2		3		4	
C[a]	T	C	T	C	T	C	T
1.2×10^{6}	6.6×10^{6}	$<10^{5}$	1.9×10^{5}	4.5×10^{8}	3.7×10^{10}[b]	$>10^{8}$	$<10^{7}$
9.6×10^{7}	8.2×10^{6}	$<10^{5}$	6.2×10^{5}	1.5×10^{11}	9.7×10^{10}[b]	4.8×10^{5}	7.6×10^{6}
1.7×10^{10}	5.3×10^{7}		2.1×105	$>10^{12}$	1.5×10^{8}[b]	7.5×10^{7}	1.5×10^{5}
8.5×10^{10}	5.7×10^{4}			3.6×10^{11}	3.3×10^{8}[c]	3.2×10^{6}	$<10^{6}$
6.6×10^{6}	7.5×10^{6}			2.0×10^{11}	2.6×10^{8}[c]	1.4×10^{6}	2.9×10^{5}
					1.0×10^{1}[c]	2.6×10^{5}	$<10^{6}$

[a] C = Control; T = Treated

[b] Pig treated with 4 ml of 987P monoclonal antibody.

[c] Pig treated with 7 ml of 987P monoclonal antibody.

Table 2-6. Numbers of viable *E. coli* in the luminal contents of 10 cm segments from pigs in litters 5-8.

LITTER NUMBER							
5		6		7		8	
C	T	C	T	C	T	C	T
$<10^{4}$	1.1×10^{6}	1.2×10^{6}	$<10^{5}$	9.9×10^{5}	2.7×10^{10}	2.4×10^{7}	4.4×10^{4}
$<10^{5}$	3.4×10^{7}	3.4×10^{6}	6.9×10^{4}	1.1×10^{8}	9.0×10^{4}	1.4×10^{6}	2.0×10^{6}
1.1×10^{6}	$<10^{6}$		2.1×10^{5}	1.8×10^{7}	3.8×10^{6}	7.4×10^{5}	$<10^{6}$
$<10^{5}$	1.7×10^{5}			NG[a]	6.8×10^{6}	3.6×10^{5}	$<10^{6}$
$<10^{5}$	7.5×10^{4}			NG	3.0×10^{5}	5.6×10^{5}	2.7×10^{6}
$<10^{5}$	$<10^{5}$				$<10^{6}$		7.4×10^{5}
5.7×10^{5}	$<10^{6}$						

[a] NG = No Growth

Table 2-7. Numbers of viable *E. coli* associated with the intestinal wall in 10 cm segments of ileum from pigs in litters 5–8.

LITTER NUMBER							
5		6		7[a]		8	
C	T	C	T	C	T	C	T
$<10^{4}$	5.1×10^{6}	NG	$<10^{2}$	1.5×10^{6}	4.8×10^{5}	6.6×10^{4}	
1.4×10^{5}	1.6×10^{6}	3.9×10^{6}	NG	1.3×10^{6}	3.4×10^{4}	1.4×10^{3}	
7.3×10^{6}	5.9×10^{4}		7.3×10^{6}	6.4×10^{5}	$<10^{2}$	1.8×10^{4}	
$<10^{4}$	NG			NG[b]	3.4×10^{5}	$<10^{5}$	
2.8×10^{5}	NG			NG	$<10^{4}$	1.0×10^{7}	
$<10^{4}$	$<10^{4}$				$<10^{6}$	2.0×10^{5}	
NG	9.8×10^{4}					2.6×10^{4}	
						3.6×10^{6}	
						$<10^{7}$	
						3.5×10^{6}	

[a] Statistically significant difference between treated and control animals ($P \leq 0.05$).

[b] NG = No Growth

Usually, intestinal wall numbers were lower than luminal content numbers.

MCA Activity in Gastric and Cecal Contents

At dilutions of 1:10, 1:100 and 1:1000 the gastric contents of the 3 treated pigs in the first study caused agglutination of 987P-positive *E. coli*. No agglutination of K88-positive *E. coli* occurred with the treated pigs' gastric contents. No agglutination with the control pigs' gastric contents occurred with 987P- or K88-positive *E. coli*.

Agglutination occurred with the cecal content supernatants of both treated pigs at dilutions of 1:10 and 1:100. Cecal contents of one treated pig also agglutinated the K88-positive strain of *E. coli* at dilutions of 1:10 and 1:100. No supernatant was obtained from the stomach contents of the treated pigs, only ropy mucus was present, so an agglutination titer was not obtained. Cecal and stomach contents of the control pig were negative for agglutination with 987P- and K88-positive *E. coli*. With the brush border adhesion test, at a dilution of 1:10 the cecal content supernatant of one treated pig was able to prevent the adhesion of 987P-positive *E. coli*, but not K88-positive *E. coli*, to brush borders. Large aggregates were seen in the brush border preparations with 987P-positive *E. coli* at a 1:10 and 1:100 dilution of the cecal content supernatant. Adhesion of 987P- or K88-positive E. coli was not inhibited at any dilution of the cecal content supernatants of the other 2 pigs.

DISCUSSION

The first 3 litters of pigs were used to determine a protective dose of 987P MCA to be administered to the gnotobiotic pigs. Results with the initial 3 litters of pigs were somewhat promising, particularly in litter 3, indicated by the prolonged survival time in the treated animals and decreased mortality rate in the animals treated with 7 ml MCA. In litter 1, the mortality rate and number of animals colonized was lower in the treated than in the control animals. Since a 7 ml dose of MCA appeared to be palliative in the third litter, 10 ml of MCA was decided upon as an adequate dose for the subsequent litters. Amounts of MCA administered in previous studies have ranged from single doses of 1 ml of a 1:12,000 titer K99 MCA for calves (Sherman et al., 1983), 20 mg of K99 MCA for calves (Sadowski et al., 1983) and 100 mg of K99 MCA for pigs (Sadowski et al., 1983) to multiple doses of 10 mg of K99 MCA (Manil et al., 1987) or 25 mg of K88 MCA for pigs (Foged et al., 1986).

The challenge inoculum varied in the first 3 litters before a reliable method of standardization of bacterial concentration was found. With an exposure to 10^{10} 987P-positive E. coli, the greatest protective effect of the

MCA as well as the most significant clinical, gross, microscopic, scanning electron microscopic and microbiologic results were obtained. However, with this number of bacteria, signs of endotoxic shock were present in the pigs. In order to remove complicating factors which might have been associated with the endotoxemia such as disseminated intravascular coagulation, the number of *E. coli* was decreased to 10^8 bacteria. With these lower numbers, however, signs of colonization were generally not present. In retrospect, it appears that a more appropriate number of the bacteria might have been in the 10^9 range since 2 control animals were colonized in the first litter which received 10^9 *E. coli*. Saunders *et al.* (1963) have reported that enteric colibacillosis will occur in colostrum-deprived, specific pathogen free pigs whether the infecting inoculum is 10^3 or 10^8 viable *E. coli*. They did not indicate whether colonization would occur with these numbers.

Initially, antibody and bacteria were orally administered to gnotobiotic pigs via a syringe placed in the backs of their mouths. However, it was difficult to determine if the pigs were receiving full amounts of the MCA and bacteria. Therefore, the MCA and bacteria were administered to the next litter of pigs via a stomach tube. However, some of the pigs developed aspiration pneumonia though, possibly from the incorrect placement of the stomach tube. Thereafter, the last 6 litters of pigs were bottle fed the MCA and bacteria. However, because of the angle and fervor with which the pigs nursed some still developed aspiration pneumonia.

Also, the first litter of pigs was challenged 2 hours after the administration of MCA compared to the last 7 litters. Since the effectiveness of protection was difficult to determine subsequent to a 2 hour time lapse, the time between challenge and treatment was shortened to 30 minutes. Challenge times after treatment in other experiments using serum, milk, colostrum or MCA have varied from 30 minutes to 26 hours, but generally ranged from one half hour to 2 hours. Unlike this study though, several of the studies continued to administer the antibody after challenge (Kohler, 1974; Kohler and Bohl, 1966; Miniats et al., 1970; Scoot et al., 1972; Svendsen and Wilson, 1971; Wilson and Svendsen, 1971).

Like previous studies (Mainil et al., 1983; Sherman et al., 1983; Svendsen and Wilson, 1971; Wilson, 1972; Wilson and Svendsen, 1971), no differences in the incidence of diarrhea were seen between the control and treated animals in the litters. All animals, both control and treated, had clinical signs of enteric colibacillosis. However, at the time of euthanasia, except for the first 3 litters, evidence indicative of colonization was not present. The single dose of MCA caused a temporary protective effect in the onset of diarrhea in litter 3 and also limited the severity of the diarrhea in the initial stages in some of the treated pigs. The onset of clinical signs in litter 3 varied from 5 hours for the control and 2 of the animals treated with 4 ml MCA to 10 hrs for all except 1 of the other treated animals. In the other litters, the onset of clinical signs was very similar. However, initially in litter 6 the diarrhea was less severe in the treated animals than the control animals.

Pigs were only observed for 2 to 3 days after challenge so the period available to determine survival time was limited. However, at the time the pigs were euthanatized they were generally recovering so a more prolonged observation period would probably have made no difference in the survival time between treated and control animals. Studies administering milk and colostrum to gnotobiotic pigs infected with *E. coli* have shown a delayed onset of diarrhea and an increased survival time (Svendsen and Wilson, 1971; Wilson, 1972; Wilson and Svendsen, 1971). In one study, K99 MCA caused a statistically significant reduction in the severity and duration of diarrhea in the treated versus control calves (Sherman *et al.*, 1983). Also using K99 MCA, Mainil *et al.* (1987) indicated that diarrhea started later and ended earlier in the treated versus control hysterectomy-derived, colostrum-deprived pigs.

Gnotobiotic pigs were used in this experiment. Jones and Rutter (1972) have shown that, even though the K88 pilus is essential for ETEC to cause diarrhea in conventional pigs, it is not required for diarrhea to occur in monoassociated gnotobiotic pigs. Therefore, a treatment directed against the pilus alone may not be effective in the prevention of diarrhea and its associated clinical signs. The effectiveness of colostrum and milk in increasing the survival time in some studies utilizing gnotobiotic pigs exposed to ETEC may be related to additional protective factors present in milk and colostrum.

Under the conditions of this study, the mortality rate ranged from 90% of the pigs in litter 3 to 0% in 5 of the 8 litters. In litters 2,4,5,6, and 8, there was no difference in the mortality rate between treated and control animals. No statistically significant difference in the mortality rate was present between the treated and control pigs in any of the litters. Mortality rates in previous passive immunization studies have differed, but generally the treated animals had a lower mortality rate than the control animals (Foged et al., 1986; Rejnek et al., 1968; Sadowski et al., 1983, 1986; Sherman et al., 1983). In litter 7, the only animal to die from the infection was a treated animal, but it was the smallest pig in the litter.

Grossly, the variable amounts of fluid present in the small and large intestines in association with liquid to semiliquid feces in the rectum correlate well with lesions associated with a hypersecretory diarrhea. The pneumonia which occurred in a large number of the piglets was probably due to the aspiration of milk while the pigs were nursing. Gastric stasis precedes the development of diarrhea in piglets (White _et al._, 1969) and this probably accounts for the milk present in the pigs' stomachs. The gastric fundic hemorrhage and hyperemic intestines present in litter 3 were probably related to an endotoxemia associated with an extremely high _E. coli_ challenge inoculum.

Microscopically, the majority of the piglets were not colonized at the time of euthanasia. Piglets in litters 1 and 3 were the only piglets with substantial evidence of adhesion histopathologically. When adhesion was present, it occurred in the lower half of the small intestine which is in accordance with what has been reported previously (Isaacson _et al._, 1978; Nagy _et al._, 1976). Bertschinger _et al._ (1972) have reported that they were unable to detect _E. coli_ using a fluorescent antibody test when the bacteria numbered less than 10^6. Also, there were few adherent bacteria observed with scanning electron microscopy.

The microscopic lesions correlated with the bacteriologic results since the animals were usually not colonized except for litters 1 and 3. No significant differences were noted between bacterial counts for the intestinal wall and contents between control and treated animals except for the wall in litter 7 and the intestinal contents in litter 3. Differences in the first 3 litters may have been significant if, at the time of death of the control piglet, a paired treated animal was euthanatized and bacterial counts were taken.

Interestingly, all of the animals had signs of diarrhea prior to euthanasia, but at the time of euthanasia, evidence of adhesion and colonization was generally not present. Therefore, it is difficult to interpret the effect of the MCA on adhesion. Theoretically, the animals could have been colonized in the early stages of the infection and have almost recovered from the infection at the time of euthanasia, usually 2 days after challenge. The MCA may have inhibited colonization in the early stages of the disease compared to the controls, but that seems unlikely since the onset and degree of clinical signs was similar in the control and treated pigs in most litters.

Theoretically, the 987P MCA would have been expected to decrease the adhesion of the 987P-positive E. coli and thus inhibit colonization. Previous reports in which serum, colostrum or milk have been administered have suggested a protective effect against enteric colibacillosis due to an antienterotoxic or antibacterial effect of the antibody. Hyperimmune serum's protective action was believed to be independent of the complement-antibody bactericidal system and was initially thought to be related to the inactivation of toxin in the intestinal lumen (Kohler and Bohl, 1966). In studies by Kohler (1967) and Miniats et al. (1970), antisera orally administered to gnotobiotic pigs infected with E. coli protected the pigs from diarrhea, but the mechanism of protection did not appear to involve a marked reduction in the numbers of live E. coli in the small intestine. They suggested that the protective effect of the antibody was due to its action on the enterotoxin. An antienterotoxic effect was not evident based on clinical signs in the present study, and furthermore would not be expected since the MCA was specifically directed against the 987P pilus. Also, gnotobiotic pigs were used in the previous studies, and since pili appear to provide no added advantage in the multiplication of E. coli in gnotobiotic pigs (Jones and Rutter, 1972), a reduction in the numbers of bacteria in treated versus control animals would probably not have been expected. A similar situation may have occurred with the gnotobiotic pigs used in this study. However, in one *in vivo* experiment where gnotobiotic pigs were given colostrum and milk, the protected pigs had 2 to 4 logs/gm lower numbers of *E. coli* than the unprotected pigs (Wilson and Svendsen, 1971). Colostrum or milk may provide additional factors, such as lactoferrin and the lactoperoxidase-thiocyanate-H_2O_2 system (Contrepois *et al.*, 1986), that are lacking in serum or MCA preparations which allow the colostrum and milk to be effective in the prevention of enteric colibacillosis in gnotobiotic pigs. Lactoferrin has been reported to have a powerful bacteriostatic effect on *E. coli* (Bullen *et al.*, 1972).

Using conventionally-reared, weaned pigs, Smith and Linggood (1971) demonstrated that the protective effect of parenterally administered *E. coli* antiserum correlated with a failure of the infecting organism, the homologous strain of *E. coli*, to proliferate in the intestine. The administration of 125 to 200 ml of antiserum usually prevented the pigs from developing diarrhea or edema disease after exposure to K88-positive *E. coli*. Protection was believed to be primarily bactericidal, but an antienterotoxic effect was also suggested. Kohler (1967) also administered *E. coli* antiserum parenterally but to gnotobiotic pigs with no reduction in the numbers of bacteria.

In a study by Smith (1972), colostrum-deprived pigs were given antiserum in milk orally and then challenged with K88-positive ETEC and a larger number of nonpathogenic *E. coli* and lactobacilli. Multiplication of homologous ETEC was inhibited in the small intestine of pigs given the antiserum. This effect, considered to be antibacterial, was believed to be responsible for delaying or preventing the onset of diarrhea. An antienterotoxic effect didn't appear to be present since antiserum against the enterotoxin did not delay the onset of diarrhea caused by *E. coli* 0141:K85ab, 88ab. Smith believed that the protective effect was associated with the inhibition of adhesion by the K88 antigen of *E. coli*.

In the initial days of a study by Mainil *et al.* (1987) the mean concentration of available K99 antigen in the feces of hysterectomy derived, colostrum deprived pigs fed K99 MCA was lower when measured with an ELISA test than in those receiving K88 MCA. Also, in a study by Sadowski *et al.* (1986), the 987P pilus levels in the feces of 987P MCA treated colostrum-deprived neonatal pigs were lower than in the control animals. Therefore, except for the study by Wilson and Svendsen (1971), studies reporting a reduction in the number of *E. coli* in the small intestine of animals receiving MCA have not used gnotobiotic pigs.

With the exception of litter 3, the 987P MCA administered did not appear to be protective against enteric colibacillosis due to 987P-positive *E. coli* in gnotobiotic pigs. Several reasons may explain the ineffectiveness of the antibody.

First, in pigs, unlike with calves, one single dose of the antibody may not be an effective treatment. Piglets are susceptible to *E. coli* infections for several weeks compared to calves and lambs which are primarily susceptible just during the first couple of days of life (Runnels *et al.*, 1980; Smith and Halls, 1967). Until calves and lambs become naturally resistant to ETEC infections, antibody need only be present for a short period of time in the intestine to prevent colonization and thus diarrhea. However with pigs, pilus-specific antibody should probably be present for an extended period of time in the intestine. Also, since antibodies in suckling pigs are constantly being diluted and lost from the small intestine, they should be continuously replaced to maintain a protective level (Kohler, 1967, 1978). Porter *et al.* (1970b) showed that even after a single large feed of milk, IgA could no longer be detected in the jejunum after 3 hours in the intestines of young piglets.

Several studies have shown that in pigs, both gnotobiotic and specific-pathogen-free, hyperimmune serum, milk and colostrum have a temporary protective effect against ETEC (Kohler, 1967, 1974; Kohler and Bohl, 1966; Miniats _et al._, 1970; Scoot _et al._, 1972; Svendsen and Wilson, 1971). In their studies, pigs were fed the antibody prior to challenge and then for 1 to 3 days after challenge at intervals ranging from 1 1/2 to 8 hours. Generally no clinical signs of diarrhea were observed during the period the antibody was administered, but following the withdrawal of the serum, diarrhea would ensue within 12 to 24 hours.

Only a slight protective effect was seen in one study where hysterectomy derived, colostrum-deprived pigs were fed K99 MCA for several days after their initial MCA feeding. All the challenged pigs developed diarrhea, and 1 pig in each group died. However, the diarrhea did start later and end earlier in the pigs fed K99 MCA compared to those fed K88 MCA (Mainil _et al._, 1987).

The previous results indicate that, in order for antibody to be effective, it must be continually present in the intestinal tract of pigs. In the preceding experiments the antibody was not only administered for a prophylactic effect but also for a treatment effect. In 1 experimental trial, a K88 MCA against all 3 serologic variants was orally administered to neonatal, colostrum deprived pigs. A therapeutic effect was noted, but there was no prophylactic effect. Pigs given a therapeutic treatment with the MCA (29 pigs), with or without a preceding prophylactic treatment, had a zero mortality rate. However, in 1 litter where the antibody was only administered prophylactically, all the animals died (Foged _et al._, 1986). In the present study, the single dose of MCA was administered in a prophylactic manner. Possibly, directly after the administration of the MCA there was inhibition of the multiplication and colonization of the ETEC, but as the antibody passed through the small intestine the bacteria were then able to colonize the intestine and cause diarrhea. If so, the antibody had only a very slight effect since clinical signs in the treated and control animals occurred at approximately the same time.

Sherman et al (1983) speculated that diarrhea occurred in calves receiving the K99 MCA because the MCA may not have completely blocked colonization. The doses of MCA administered in the present study may have been insufficient and thus colonization with subsequent clinical signs of enteric colibacillosis was able to occur. Isaacson _et al._ have said that, even if only 10% of a K99-piliated strain of _E. coli_ was capable of adhering, the bacteria would be able to proliferate to a high enough concentration such that > 10^9 bacteria could adhere to 10 cm of ileum 16 hours after inoculation of the pig with 10^9 bacteria. Consequently, the MCA would have to be present in a high enough concentration to prevent more than 90% of the piliated bacteria from binding.

The type of antibody used in this study, IgG, may explain the ineffectiveness of treatment. It's generally believed that IgG is of minor importance compared to IgA and IgM in the protection of the intestine (Bienstock, 1975; Miler et al., 1975; Sellwood, 1984b). Porter et al. (1970a) have suggested that secretory IgA is the first line of defense against E. coli infections in pigs. Secretory IgA is highly resistant to proteases and probably persists in the intestine longer than IgG and IgM. Miler et al. (1975) determined the protective effect of porcine colostrum, serum, IgG, IgM and IgA against an enterotoxic strain of E. coli in newborn germfree pigs. They reported that IgA isolated from immune porcine sera and colostrum was effective at a lower concentration against ETEC in ligated pig loops than IgG or IgM isolated from the same sources. With the brush border adhesion inhibition assay, IgG isolated form colostrum and milk was incapable of blocking the binding of the K88 pilus to the intestinal brush border surface, unlike IgA and IgM (Sellwood, 1984b). The use of an IgA type of 987P MCA may have been more effective in the treatment of enteric colibacillosis in the pigs.

Some MCAs are very susceptible to changes in pH and salt concentrations (Goding, 1986). The antibody may not have been effective because of its early destruction in the gastrointestinal tract due to gastric pH or proteases. An acidic gastric pH is capable of destroying or altering antibody. However, the gastric pH of newborn pigs is relatively high (Moon, 1974; Smith and Jones, 1963). Also, proteolytic enzymes within the stomach may not be active in newborn piglets. The pH of the gastric contents does not drop low enough soon after birth for the enzymes to be strongly hydrolytic. Additionally, milk rapidly escapes into the intestine within 5 minutes of being suckled, possibly not allowing adequate time for the globulins to be destroyed in a significant amount. In the present study 987P MCA present in the gastric contents of 3 treated pigs was still active in a slide agglutination assay, as well as MCA present in the cecal contents of another pig.

Germfree animals in comparison to conventional animals do, however, have an increased number of enzymes in their intestinal contents (Kawai and Morotomi, 1978). Two explanations have been postulated for the increase. Slower peristalsis of the germfree intestine may lead to slower elimination of intestinal contents, or a slower turnover of intestinal epithelial cells may result in more mature cells with more active synthesis of certain enzymes (Savage et al., 1981; Wostmann and Bruckner-Kardoss, 1981). The higher concentration of proteolytic enzymes would thus lead to a more rapid destruction of intestinal Igs (Fubara and Freter, 1972). The rapid proteolytic destruction would thus lead to the ineffectiveness of the MCA, particularly if the MCA was not replenished on a regular basis.

Using a brush border adhesion test in the present study, 1 pigs' cecal content supernatant at a dilution of 1:10 was able to prevent the adhesion of 987P-positive E. coli. Large aggregates of bacteria were seen at the 1:10 and 1:100 dilution, even though adhesion was not inhibited at 1:100. The presence of the aggregates tends to indicate that the antibody was present and capable of agglutination. In the other treated pig adhesion to the brush border was not inhibited at a 1:10 dilution. Therefore, in at least some instances antibody appears to be active as it passes from the small intestine and capable of preventing adhesion in an in vitro situation.

The titer of the antibody used in the present study was determined with a slide agglutination test which may not have been a good indicator of the protective ability of the antibody. Nagy et al. (1978) and Rutter et al. (1976) have observed that antibodies demonstrated by agglutination tests are not major contributory factors in protection. Nagy et al. (1985) noted no correlation between colostral or seroagglutinins to fimbrial antigens of E. coli and mortality rates in litters challenged with homologous pili, but good correlation was found between colostral precipitins to K88 antigens and mortality rates in litters. The effect of vaccination on antipilus antibody titers differed from almost nil (K99) to slight (K88 ab and ac) or marked (987P). However, no correlation was noted between these titers and mortality rates in litters challenged with homologously piliated _E. coli_.

Another factor which may have caused the MCA's ineffectiveness is the binding strength of the antibody. Some MCA show weak epitope binding (DePinho _et al._, 1986; Young, 1985). If the MCA had a low affinity, gut peristalsis could have disassociated the pilus-MCA complex. Some environmental conditions may alter the MCA binding site and thus prevent the MCA from binding (Goding, 1986). Also, the species of origin of the MCA may have limited the antibody's effectiveness. According to Raybould (1985), in some instances passive immunity may be induced more effectively by MCA originating from the same species as the animal under treatment.

The pilus subunit is composed of numerous epitopes. Monoclonal antibodies against some pilus epitopes are capable of preventing adhesion whereas others are not (Schifferli _et al._, 1987). _In vivo_, the 987P MCA may not have been able to prevent adhesion because of the epitope it was directed against. A MCA directed against another pilus epitope or several different MCAs directed against multiple pilus epitopes might be more effective in the prevention of adhesion.

Alternate mechanisms other than the direct inhibition of adhesion via which the antibody could have prevented the bacteria from colonizing the gut may involve agglutination, opsonization, bactericidal or bacteriostatic effects, steric hindrance, altered bacterial surface charge or plasmid curing (Brinton, 1978; Linggood and Porter, 1978; To et al., 1984). However, if the principal mechanism was via antibody opsonization or agglutination, a protective effect would be expected from antibodies to any pilus present on infecting bacteria, even if that pilus was not necessary for virulence (Brinton et al., 1983). Jayappa et al. (1983) concluded that both mechanisms probably occur in the protection of pigs.

The type of experimental animals, gnotobiotic pigs, used in this study may have also affected the results. In monoassociated gnotobiotic pigs, colonization can occur with most bacteria, and the virulence factors required for colonization in a conventional animal may not be necessary. In fact, even when some animals are immunized against certain bacteria, the bacteria are still able to colonize the intestine (Shedlofsky and Freter, 1974). Several factors contribute to the ease with which gnotobiotic pigs are colonized in the intestine: 1) lack of local and systemic immune mechanisms (rev. Kim, 1981), 2) the lack of bacterial antagonism (Aly and Shinefield, 1982; Dubos, 1963; Freter, 1956; van der Waaij *et al.*, 1971), 3) slowed peristalsis. Jones and Rutter (1972) have shown that, even though the K88 pilus is essential for ETEC to cause diarrhea in conventional pigs, it is not required for diarrhea to occur in monoassociated gnotobiotic pigs. It was assumed that the slower intestinal motility and more rapid growth of the monoassociate in the absence of competition from other microflora obviate the need for ETEC to adhere to the intestinal wall. Therefore, as appeared to be the case in the present study, *E. coli* could cause diarrhea without significant adhesion in gnotobiotic pigs. In ligated intestinal loops, where the washout effects of intestinal motility are absent, ETEC caused fluid accumulation in the absence of extensive adhesion to the small intestinal epithelium. Also, ETEC neither multiplied faster nor attained higher total numbers than nonenterotoxigenic *E. coli* (Bertschinger *et al.*, 1972). Thus, extensive adhesion is not essential for *E. coli* to cause fluid loss by the small intestine and therefore, diarrhea could have occurred in the gnotobiotic pigs without extensive adhesion. In fact, that appeared to be the case in the present study. Clinically, the animals had signs of diarrhea but evidence of colonization was not present in most pigs. Since colonization was limited in most litters, the value of the antibody was difficult to determine. Several reasons may exist to explain the lack of adhesion.

One reason for the lack of colonization may be as simple as an inadequate challenge inoculum, since with an inoculum of 10^{10} E. coli, adhesion and colonization were present. Another reason for the lack of adhesion may be associated with genetic resistance to 987P-positive E. coli. However, Moon et al. (1979) have reported that they have not encountered pigs genetically resistant to 987P-positive E. coli and also, in the previous chapter, all the brush border samples examined had receptors for 987P-positive E. coli. The possibility does exist, though, that some pigs may be genetically resistant to 987P-positive E. coli since neither the control nor treated pigs were successfully colonized.

Another reason for the lack of adhesion may be due to a lack of a capsule by the strain of E. coli used. Some reports indicate that the capsule enhances the colonizing ability of E. coli strains that produce them (Hadad and Gyles, 1982; Nagy et al., 1976; Smith and Huggins, 1978). Piliated acapsular mutants of many strains have been shown to colonize the small intestine to a smaller extent than the encapsulated parenteral strains. Isaacson et al. (1977) reported that both a polysaccharide capsule and the 987P pilus are required for colonization by strain 987 to occur *in vivo*, even though the acapsular piliated form of strain 987 adhered to isolated epithelial cells *in vitro*. Moon *et al.* (1979) have suggested that the capsule could be required for the expression of pili in vivo or that both structures could be required for adhesion by strain 987 *in vivo*. Moon and Runnels (1984) indicated that an encapsulated ETEC strain was more virulent in suckling neonatal pigs than was its acapsular mutant. In the present study, the bacteria were able to adhere in the first 3 litters. Unless the bacteria lost the ability to produce the capsule, this seems to be an unlikely explanation. However, it is interesting to note that in the first litter, a challenge inoculum of 10^8 bacteria was able to cause colonization of the small intestine. However, in the last 4 litters challenged with 10^8 *E. coli*, only 1 animal was colonized. Evidence of colonization was present bacteriologically in that animal but not histologically.

In gnotobiotic animals, glycoproteins that often act as receptors for bacterial adhesion are of a different composition because they are not degraded by indigenous microflora (Jones, 1977). Thus, the receptors for the 987P pili in the present study may be incapable of binding piliated *E. coli*. However, this possibility does not seem likely since in some pigs adhesion was present.

Even though bacterial adhesion was not seen in most of the intestinal sections, there was a close association of the bacteria with the small intestinal villi. Bacteria were often seen entrapped in mucus adjacent to the villi. The mucus may have provided binding sites for the *E. coli*, and along with the decreased gut peristalsis in the gnotobiotic pig, multiplication of the bacteria may have been great enough to produce a sufficient amount of enterotoxin to cause diarrhea. Freter (1981) has suggested that to resist the flux of intestinal secretions and to colonize tissue, it may be sufficient for bacteria to bind to mucus alone.

In a recent study by Broes *et al.* (1988) it was shown that a diarrhea-causing ETEC, strain 08:KX105, colonized the neonatal pigs' small intestine to a moderate extent but did not strongly adhere to the intestinal epithelium. The *E. coli* strain was classified as a nonclassical ETEC which produced LT and ST_b enterotoxin and usually produced 987P pili. *Escherichia coli* small intestinal colony counts of 10^8 or 10^7/10 cm were present in the ileum and jejunum respectively of all piglets with diarrhea. However, with histology, immunofluorescence microscopy and scanning electron microscopy little evidence of bacterial adhesion to the small intestinal epithelium was seen. Only a few adherent bacteria were seen on the tips of the villi or randomly scattered in the lumen. An association index of 1.1 was found. In the present study in the last 5 litters, little evidence of adhesion was present on the histopathologic and scanning electron microscopic sections of intestine. It is interesting to note, that in the initial part of this study, an indirect fluorescent antibody test was performed on the first 3 litters with little evidence of positive fluorescence even though the animals were colonized microbiologically. Use of the test was subsequently discontinued since there appeared to be a technical problem with the test. Yet, year-old positive samples from a previous study were positive using the same technique. Broes _et_ _al._ (1988) suggested that the 08:KX105 strain lacked the ability to strongly adhere to the intestinal mucosa, possibly only binding to mucus. Also, unlike the classical 987P-positive _E. coli_ strains which produce ST_a enterotoxin and are usually isolated from pigs less than 1 week old, the ETEC used in their study produced LT and ST_b enterotoxin and were isolated from weaned as well as suckling pigs. They speculated that the 987P pili produced by such strains was modified leading to a decreased ability to adhere to intestinal villi _in vivo_. They thus demonstrated that certain ETEC may cause acute diarrhea in piglets in the absence of extensive colonization of the small intestine. The serotype of the _E. coli_ strain used in the present study is unknown but many parallels can be drawn between the *E. coli* strain used in my study and that previously described.

The results of the present study suggest that the direct passive oral immunization of gnotobiotic pigs with 987P MCA (from hybridoma 4A3) is not beneficial in the prevention of diarrhea caused by 987P-positive *E. coli* in gnotobiotic pigs. No significant differences in morbidity, mortality, gross lesions, histologic lesions and most of the bacteriologic counts were present between the treated and control animals in the 8 litters. Since pigs infected with 10^8 *E. coli* had clinical signs of diarrhea even though histologic, scanning electron microscopic and microbiologic results generally indicated that colonization was not present, it was difficult to determine the effectiveness of the antibody.

Though the MCA was ineffective in gnotobiotic pigs, the efficacy of the antibody may differ in conventional animals. Therefore, in the future it may be worthwhile to determine the MCA's effect in conventional pigs. Also, *in vitro* and *in vivo*, it would be interesting to determine the antiadhesive ability of the MCA in comparison to colostrum, milk, a monospecific polyclonal serum and a mixture of several different 987P MCAs. Additionally, the 987P MCA could be used in the development of various immunodiagnostic tests, such as a fluorescent antibody test or an ELISA, for the clinical detection of 987P-positive _E. coli_.

SUMMARY

This study was used to determine if the clinical signs, lesions and _in vivo_ adhesion of 987P-positive _E. coli_ to the intestinal mucosa of gnotobiotic pigs could be altered by the oral administration of 987P MCA. Two to 5-day-old gnotobiotic pigs from 8 litters were each given 0.5 to 12 ml of 987P MCA, challenged 0.5 to 2 hours later and then observed clinically for signs of enteric colibacillosis. Gross, histopathologic, scanning electron microscopic and microbiologic examinations were performed.

Clinical signs, bacteriologic counts, and gross, histologic and scanning electron microscopic lesions were similar in the control and treated animals. Clinically, all except two animals had diarrhea. Gross lesions were minimal and variable, consisting primarily of fluid in the small and large intestines and pasty to liquid rectal contents. Adhesion and colonization based on microbiologic, histologic and scanning electron microscopic examinations were limited. Only animals in litters 1 and 3 had substantial adhesion and colonization.

Based on the results of this study the 987P MCA was ineffective in the prevention of diarrhea in gnotobiotic pigs challenged with 987P-positive _E. coli_. An accurate assessment of the MCA's effect on adhesion was difficult, though, because of the limited colonization of both the treated and control animals. It was also difficult to assess the efficacy of the 987P MCA because gnotobiotic pigs were used in the study and reports have indicated that pili are not a necessary virulence attribute for ETEC in gnotobiotic pigs.

CHAPTER 3

THE EFFECT OF THE ORAL ADMINISTRATION OF 987P MONOCLONAL ANTIBODY ON ENTEROTOXIN PRODUCTION OF 987P-POSITIVE *ESCHERICHIA COLI* IN GNOTOBIOTIC PIGS

CHAPTER 3

THE EFFECT OF THE ORAL ADMINISTRATION OF 987P MONOCLONAL ANTIBODY ON ENTEROTOXIN PRODUCTION OF 987P-POSITIVE *ESCHERICHIA COLI* IN GNOTOBIOTIC PIGS

INTRODUCTION

The second virulence determinant necessary for ETEC to cause diarrhea in neonatal pigs is enterotoxin production (Smith and Halls, 1967). Two types of enterotoxins, based on their thermolability, antigenicity and molecular weight, are produced by ETEC: 1) ST and 2) LT. In the present study, 987P-positive *E. coli* were used. Strains of ETEC which produce the 987P pilus are always associated with ST enterotoxin production (Guinee and Jansen, 1979b; Moon *et al.*, 1980).

Two distinct types of ST are known: 1) ST_a which is methanol soluble, and active in the infant mouse model and in ligated jejunal segments of piglets 1-3 days old and 2) ST_b which is methanol insoluble, inactive in the infant mouse model and active in ligated intestinal segments of 7-9-week-old weaned pigs (Burgess *et al.*, 1978; Gianella, 1976; Kapitany *et al.*, 1979a,b; Newsome *et al.*, 1978a,b).

The action of ST_a is almost instantaneous, relatively short lasting and readily reversible by rinsing (Evans et al., 1973; Field et al., 1978; Hughes et al., 1978; Guerrant et al., 1980). The ST_a toxin binds to intestinal membrane brush border receptors (Frantz et al., 1984; Giannella et al., 1983). The mechanism of action of ST_a involves the activation of guanylate cyclase in the small intestinal epithelial cell with a subsequent increase in cGMP levels (Field et al., 1978; Hughes et al., 1978; Newsome et al., 1978a,b). The toxin causes a hypersecretory diarrhea by decreasing NaCl absorption and increasing chloride secretion (Guandalini et al., 1982; Rao et al., 1980).

The ST_b toxin's mechanism of action is believed to be cyclic nucleotide independent (Kennedy et al., 1984). Like ST_a, ST_b has a rapid, but reversible, onset of action. In vivo, ST_b causes a significant increase in the amount of bicarbonate, sodium and chloride in the intestinal contents (Weikel et al., 1986).

Assays for the detection of ST are limited because of its nonantigenic nature, and the more commonly used tests involve animals (Dean et al., 1972). Recently, a few immunologic tests for the detection of ST have been described (de Mol et al., 1983, 1985; Dreyfus et al., 1983; Thompson et al., 1984).

Sherman *et al.* (1983) observed that calves to which K99 MCA was administered and subsequently challenged with K99-positive *E. coli* all had diarrhea. They speculated that the number of *E. coli* in the challenge inoculum might have been large enough to produce sufficient enterotoxin without adhesion to the mucosa to cause diarrhea. In the present study, the *E. coli* inoculum was washed prior to being given to the pigs to remove the enterotoxin. The objective of this study was to determine the effects of 987P MCA on enterotoxin production in gnotobiotic pigs challenged with 987P-positive *E. coli*.

MATERIALS AND METHODS

Experimental Animals

Piglets from the last two litters, 7 and 8, were used in the this study. The experimental protocol was the same as that previously described in Chapter 2 with the following additions.

cGMP Radioimmunoassay

Piglets were anesthetized with sodium pentobarbital[a]. An incision was made on the ventral abdominal surface of the pigs. The small intestine was removed, and a 3-cm section of ileum near the cecum was opened and excised. The ileal section was then immediately placed on ice and

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[a] Butler Company, Columbus, OH.

the mucosa stripped from the intestine with a razor blade. The mucosa was then homogenized within 60 seconds of tissue removal in 5 ml of 6% TCA[b] with a Teflon serrated-tipped tissue grinder[c] rotating at approximately 900 rpm at 4°C. The protein concentration of 1 ml of the TCA suspension was determined by the Lowry method (refer to Chapter 1). The remaining part of the suspension was centrifuged at 10,000 x g. The TCA was removed from a 2 ml volume of the supernatant solution with 3 2-ml washings of diethyl ether[b]. The 3 ether washings were pooled. The TCA-free extract, the ether layer, was dried with a nitrogen evaporator[d] and then reconstituted to 2 ml with 50 mM acetate buffer. The samples were then frozen at -70°C until the test was performed. The cGMP was measured using a commercial radioimmunoassay kit[e]. Three dilutions of the sample were used. The sample dilutions were run in duplicate and counted in a scintillation counter[f] for 5 minutes per sample (Kapitany et al., 1978).

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[b] Mallinckrodt Inc., Paris, KY.

[c] Thomas Co., Philadelphia, PA.

[d] Model 111, Organomation Assoc., Inc., Shrewsbury, MA.

[e] Amersham Corporation, Arlington Heights, IL.

[f] Beckman Model LS 3133P, Beckman Instruments, Inc., Palo Alto, CA.

Suckling Mouse Assay

A section of ileum, 30 cm in length, was removed from each pig. The intestinal contents were rinsed from the lumen with 5 ml of PBS and placed in a tube on ice. The contents were centrifuged at 5,000 x g (4°C) in a refrigerated centrifuge[g] for 15 minutes. The supernatant was then filtered through a 0.20 µm filter. The supernatant was then frozen at -70°C for 1 to 2 days.

Heat stable enterotoxin activity was then determined using a suckling mouse test. As a positive control, the 987P-positive strain of _E. coli_, strain 81-1469, was grown in 10 ml trypticase soy broth[h] overnight at 37°C in a shaking water bath[i]. The culture was then filtered through a 0.20 µm filter. Intestinal content supernatants were thawed. One drop of methylene blue dye was added to the broth and intestinal content filtrates to determine if the injection had been properly performed. Three-day-old mice were separated from their mothers. The mice were then given 0.1 ml of broth or intestinal content filtrate. Three mice were used for each sample. Using a syringe and a 30 gauge hypodermic needle, the fluid was injected through the body wall directly into the milk-filled

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[g] International Refrigerated Centrifuge, Model PR-6, International Equipment Co., Needham, MA.

[h] Baltimore Biological Laboratories, Cockeysville, MD.

[i] Precision Scientific Co., Chicago, IL.

stomachs of the mice. The mice were kept at room temperature for 4 hours and then euthanatized with ether. The abdomen of each mouse was then opened, and the entire intestine was removed. Mice with methylene blue in the peritoneal cavity were not used. The intestine and the remainder of the body were then weighed separately. Gut weight to body weight ratios below 0.070 were considered negative for enterotoxin, ratios between 0.070 and 0.090 were considered equivocal and ratios above 0.090 were considered strongly positive for enterotoxigenic activity (Dean *et al.*, 1972; Gianella, 1976).

Statistical Analysis

Data were analyzed using a paired t test to determine if differences existed in cGMP values between control and treated animals. A Chi-square test was done to determine differences in ST_a between control and treated animals using the suckling mouse assay. Differences between groups were considered significant at the $P \leq 0.05$ level (Steel and Torie, 1980).

RESULTS

cGMP Radioimmunoassay

Results of the cGMP RIA are listed in Table 3-1. No statistically significant difference was present between the treated and control cGMP values for the animals in litters 7 and 8. Values ranged from 2.0 to 3.7 picomole

Table 3-1. Cyclic GMP radioimmunoassay results from ileal samples of gnotobiotic pigs challenged with 987P-positive *E. coli*.

Litter Number[a]		pmole cGMP/mg protein					
7	C	2.0	2.2	3.0	3.3	-	
	T	2.7	2.8	2.8	3.1	3.7	3.0
8	C	3.5	3.3	2.6	2.3	2.5	
	T	3.4	3.2	3.1	2.7	2.4	

[a] C = Control; T = Treated.

(pmole)/mg protein with an average value of 2.7 for the control animals and 3.0 for the treated animals.

Suckling Mouse Assay

Intestinal filtrates from both the control and treated animals in the 2 litters were all negative for ST^a enterotoxin. Results for the 987P-positive E. coli broth culture were in the equivocal range (Table 3-2).

DISCUSSION

No statistical difference was present in cGMP levels between the treated and control animals in the 2 litters. The cGMP level was only slightly elevated compared to reported control values, 2.3 ± 0.1 pmole/mg protein (Newsome et al., 1978a,b). Values for the 2 control animals in litter 7 with no diarrhea were very similar to the values in animals with diarrhea. Average cGMP values in the treated pigs were slightly higher than the control animal cGMP values. Since diarrhea was lessening at the time of euthanasia, ST production was probably low. The 2-day observation period after challenge was probably too long for an accurate assessment of an elevations in cGMP. However, euthanasia of the animals prior to this time would have greatly limited the useful data obtained from the last two litters. In retrospect, sacrifice within 2 hours of the onset of clinical signs would probably have been most beneficial in

Table 3-2. **Suckling mouse assay results from intestinal filtrates of gnotobiotic pigs challenged with 987P-positive *E. coli*.**

Litter Number[a]		Gut Weight/Body Weight[b]					
7	C	0.054	0.057	0.063	0.063	0.055	
	T	0.062	0.060	0.062	0.068	0.065	0.063
	E. coli	0.085					
8	C	0.068	0.068	0.061	0.059	0.058	
	T	0.062	0.069	0.059	0.057	0.062	
	E. coli	0.087					

[a] C = Control; T = Treated; *E. coli* = 987P-positive *E. coli* broth culture .

[b] Ratio is the average of 3 mice.

the demonstration of cGMP levels, especially since Holmgren (1985) has suggested that diarrhea itself may reverse the ST action by "diluting" the toxin from its mucosal receptor. In one report, biopsies were taken from mice 40 minutes after dosing with toxin to determine cGMP levels (Newsome, 1978a).

A report by Kapitany _et al._ (1978) indicated that cGMP was not elevated until an ED (minimum dose of protein required to give a positive response in suckling mice) dose level of 1000, where fluid secretion was most pronounced, was given. Some authors have indicated that there is a change in the ratio of cAMP to cGMP so examining the ratio of cAMP to cGMP may have been beneficial. Using freeze-clamped, full-thickness intestinal biopsies, Newsome _et al._ (1978a,b) said that ST_a raised the cGMP concentration and lowered the cAMP\cGMP ratio in mice.

Intestinal samples were removed and placed on ice as soon as possible, but post mortem changes may have still occurred and altered the cGMP levels. Also, because of time constraints, samples for the cGMP RIA were frozen for several days prior to performance of the test which may have adversely affected the results.

Since the action of ST_a is reversible by rinsing, it was postulated that ST_a bound in the piglet small intestine could be "rinsed" free and subsequently detected in the suckling mouse assay. Results of the assay indicated that enterotoxin was not present in an amount large enough to cause intestinal secretion in suckling mice. Removal of the luminal contents with 5 ml of PBS may have diluted the enterotoxin to a level to low to cause fluid secretion. Also, diarrhea was abating at the time of euthanasia so the amount of bound ST_a may have been low, particularly since ST_a's action is almost instantaneous and relatively short lasting (Field *et al.*, 1978; Hughes *et al.*, 1978; Guerrant *et al.*, 1980). Freezing the intestinal filtrate samples should not have affected the results since Dean *et al.* (1972) froze toxin at -70°C for 18-20 days with no adverse effect on the enterotoxins' activity.

The gut weight/body weight ratio for the 987P-positive *E. coli* broth culture was in the equivocal range for enterotoxin production (0.070 to 0.090, Dean *et al.*, 1972; Gianella, 1976). Moon *et al.* have stated that a gut to carcass weight ratio value $\geq$ 0.085(pmole)/mg is considered positive, and in that case the cultures would be considered positive for ST_a production. The low level of ST production may be associated with the method of culture. It has been suggested that ST_a production by *E. coli* strains isolated from animals other than humans may not be maximal in trypticase soy broth (Scotland *et al.*, 1985). Alderete and Robertson (1977) have indicated that the production of ST is relatively low in aerated cultures and have suggested that a synthetic medium, casamino acids-yeast extract medium, be used. Also, the ability of *E. coli* strains to secrete ST_a toxin is occasionally lost on subculturing *in vitro* (Burgess *et al.*, 1978; Dean *et al.*, 1972).

Since cGMP was only slightly elevated in this study and the 987P-positive *E. coli* broth culture suckling mouse test results were in the equivocal range the 987P-strain of ETEC used in the study may be an STb producer. If so results from this chapter, lack of elevation of cGMP, and Chapter 2, lack of adhesion of 987P-positive *E. coli* to brush borders would correlate well with the results of Broes *et al.*, 1988. Broes *et al.* reported that a ST_b producing, 987P-positive strain of *E. coli* colonized the neonatal pigs' small intestine to a moderate extent but did not adhere to the intestinal epithelium.

SUMMARY

This study was used to determine if enterotoxin production in gnotobiotic pigs challenged with 987P-positive E. coli could be affected by the oral administration of 987P MCA. The cGMP content of the pig ileal mucosa was determined using a cGMP radioimmunoassay. No statistically significant difference in cGMP values was present between the treated and control animals in the two litters. Values ranged from 2.0 to 3.7 pmole/mg protein with an average value of 2.7 for the control animals and 3.0 for the treated animals.

Intestinal content filtrates from the piglets were used in a suckling mouse assay for the detection of ST_a in luminal contents. All intestinal content filtrates were negative for ST_a in the assay.

Based on the results of this study, at the time of euthanasia, substantial amounts of enterotoxin were not present in the piglets' small intestines. Diarrhea had abated by the end of the two day observation period, so an accurate assessment of enterotoxin production in this study is difficult.

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