The influence of some microbial and host factors on the pathogenesis of enterotoxigenic Escherichia coli infections

Paul Lewis Runnels
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The influence of some microbial and host factors on the pathogenesis of enterotoxigenic *Escherichia coli* infections

by

Paul Lewis Runnels

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Veterinary Pathology

Approved:

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REVISION OF TABLE 2, MANUSCRIPT 3, WITH COMMENTS ON SIGNIFICANCE OF THE RATIOS OF AFFECTED SAMPLES TO TOTAL SAMPLES

TABLE 2

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GENERAL INTRODUCTION

Severe, watery diarrhea caused by enterotoxigenic Escherichia coli (ETEC) tends to be restricted to young (less than 1 week old) animals. The specificity of the restriction is related to both the strain of ETEC and the host animal. Thus, ETEC which express the K99 pilus antigen are associated with diarrheal disease in calves and pigs up to a few days old (111, 148) while ETEC which express the K88 pilus antigen are associated with diarrheal disease of pigs, but not calves, up to 6 weeks old (111). Pilus antigens (K88, K99, 987P, F41) effect adherence of ETEC to small intestinal epithelium. This adherence overcomes the tendency of the small intestine to purge the ETEC into the cecum and colon. Thus, pilus mediated adherence facilitates intense colonization of the small intestine by ETEC. Properties of ETEC in addition to the pilus antigens are important in causing diarrheal disease. These include the secreted enterotoxins (STa, STb, and LT) and capsular antigens (66, 109, 151, 184). Enterotoxigenic E. coli which express both capsular antigens and adhesive pilus antigens are more virulent and colonize the small intestine more intensively than do ETEC which express one, the other, or neither (151).

Although 2-day-old calves are resistant to challenge with ETEC, epidemiologic evidence suggests that ETEC are associated with diarrheal disease in older calves (1, 107). The epidemiologic evidence (1, 107, 115) also lends support to the commonly held belief that a significant fraction of neonatal diarrhea in animals is caused by mixed infections. Agents associated with mixed infections in older calves include ETEC,
rotavirus, Cryptosporidia sp., and coronavirus (1, 107, 115). However, until recently, experimental evidence of the importance of mixed infections as causes of diarrheal diseases of neonatal animals has been scant and not well tested (58, 102, 159, 173, 184).

The studies reported here focus on the influence of host and microbial factors on the pathogenesis of ETEC diarrheal disease of neonatal animals. First, the influence of host age is examined by analyzing adherence of piliated ETEC to isolated intestinal epithelial cells of calves, pigs, and mice which varied in age from 2 days old up to adults. Second, the effect of a microbial factor (expression of a mucoid, type A capsule) is examined by analyzing adherence of encapsulated, K99+ ETEC to isolated intestinal epithelial cells of 2- to 5-day old pigs. The third and fourth reports examine both microbial and host factors in experiments testing how E. coli-inoculated calves were affected by concomitant infection with rotavirus, age of the calf at time of inoculation, amount of milk fed the calf, length of time the calf was observed after inoculation, dose of ETEC, strain of E. coli, and strain of rotavirus. The first four factors are related primarily to the host while the last three are microbial. The experiments were planned to identify existence of interaction between the E. coli and rotavirus and, if there was significant interaction, to replicate the experiments and identify factors contributing to the interaction. Our planned studies differed from previous reports (58, 102) in our commitment to consistently reproduce and study severe diarrheal disease
in calves about 1 week old, as has been reported to occur in field cases (1, 107).

This dissertation has been prepared in the alternate format and includes 4 manuscripts, three of which have been published. It is written in the style of the journal *Infection and Immunity*. The first manuscript "Development of resistance with host age to adhesion of K99⁺ *Escherichia coli* to isolated intestinal epithelial cells" was published in *Infection and Immunity*, Vol. 28, pp. 298-300, 1980. The second manuscript "Capsule reduces adherence of enterotoxigenic *Escherichia coli* to isolated intestinal epithelial cells of pigs" was published in *Infection and Immunity*, Vol. 45, pp. 737-740, 1984. The third manuscript "Interaction of rotavirus and enterotoxigenic *Escherichia coli* (ETEC) in gnotobiotic calves" was published in the proceedings of the Third International Symposium on Neonatal Diarrhea, S. D. Acres, ed. Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, pp. 343-358, 1981. The fourth manuscript "Effect of altering microbial and environmental variables on the response of gnotobiotic calves to dual infections with rotavirus and *Escherichia coli*" has not been published, but is being prepared for submission to the American Journal for Veterinary Research. These studies were planned and carried out and manuscripts written principally by the Ph.D. candidate, Paul L. Runnels, with advice and approval by the major professor, Dr. Harley W. Moon, and other coauthors.

A literature review precedes the first manuscript. A general summary and discussion follow the last manuscript. Literature for the
introduction, literature review, and general summary and discussion are cited separately in an additional literature section (p. 139) which follows the general summary and discussion. Literature cited in a manuscript are included at the end of that manuscript.

Typographic errors and editorial oversights which occurred in manuscripts already published are corrected in the text. Substantive changes which were suggested by the graduate committee are included in appendices referable to the appropriate manuscript.
LITERATURE REVIEW

Any infectious disease results from interaction since an appropriate pathogen must interact in an appropriate manner with an appropriate host to cause disease. This dissertation examines several factors which may affect the interaction between enterotoxigenic Escherichia coli (ETEC) and neonatal animals. The factors include age of the host, concomitant infection of the host with rotavirus, and virulence attributes of the ETEC.

Infections with enterotoxigenic Escherichia coli ETEC cause severe, watery, often fatal diarrhea in many mammals, including humans, calves, lambs, and pigs (3, 40, 59, 79, 89, 104, 111, 114, 139, 144, 148, 160, 176a). ETEC strains tend to be highly host-species specific and host-age specific (111, 148, 173). Among food animals, susceptibility to diarrheal disease due to ETEC is usually confined to the neonatal and post-weaning periods.

ETEC, unlike the harmless, autochthonous E. coli of the intestinal tract, have specific characteristics called virulence attributes which enable them to colonize the small intestine of susceptible hosts and stimulate the mucosal epithelium to secrete copious watery fluid. The virulence attributes include secretion of enterotoxin and expression of colonization factors.

Enterotoxins are plasmid encoded proteins or peptides which are secreted into the environment of the ETEC (145, 147, 149, 150). Enterotoxins are classified primarily on their susceptibility to heat, so that there are heat labile (LT) and heat stable (ST) enterotoxins.
LT, a highly antigenic protein is both cell associated (22) and free in culture supernatant fluid and was originally defined as having its activity destroyed by heating at 65°C for 15 minutes (147). *Escherichia coli* LT is closely related to cholera toxin (CT) in both structure and activity (73). Like CT, it is composed of subunits (26, 27). The "B" subunit is the portion which binds to the host cell membrane and is composed of five similar peptides (55). Among LT of *E. coli* from different species, the B subunit varies slightly (23, 52, 74). The A' subunit is the toxic moiety and transfers into the cell membrane, stimulating the cyclic AMP (cAMP) system to cause secretion of chloride-rich, watery fluid (44, 70, 84). The LT can be detected by radial passive immune hemolysis (10), GM1 ganglioside ELISA (163), and more commonly by biologic assays for its activity. The most commonly used biologic assays are alteration of cultured cells such as Y-1 adrenal cells (33) and the Chinese Hamster Ovary (CHO) cells (60). Additionally, live cultures, bacterial lysates, or culture supernates can be placed into intestinal loops of live animals and cause dilation of the loops with fluid (17, 65, 110, 112, 113). Preincubation of the LT with antiserum against either the holotoxin or its subunits results in neutralization of its toxic biologic activity.

*Escherichia coli* ST, in contrast to LT, is a low molecular weight peptide which mediates secretion of watery fluid via cyclic GMP rather than cAMP (53, 61, 87, 91, 134). ST secreted by human strains of *E. coli* differs slightly genetically and in amino acid sequence from ST secreted by animal strains of *E. coli* (120a), but their mechanisms of
action are similar. These human and bovine ST toxins are referred to as STa [also as ST Ia (human) or ST Ib (bovine)]. The STa toxins are methanol soluble and stimulate net secretion in both the infant mouse and neonatal pig small intestine, but not in 7- to 8-week-old pig small intestine (16). However, STa is active in both 7- and 14-week-old pigs as evidenced by its ability to inhibit net fluid absorption in pigs that age (176b). Additionally, there is a subset of ST toxins which are referred to as STb or ST II (16). STb is methanol insoluble and is inactive in the infant mouse assay but active in 7- to 8-week-old pig intestine and in rabbit intestine (16). The mechanism of action of STb is unknown, but secretion is not mediated via either the cAMP or cGMP system. The ST toxins are poorly antigenic, consequently traditional assays for the toxin depend on detecting biologic activity (28, 89, 105, 123, 147). More recently, ST toxins (STa) have been coupled to antigenic proteins such as bovine albumin (50a, 86), bovine IgG (54), and E. coli LT (88). Anti-ST antibody against some of the haptenized antigens have been used to develop radioimmunoassays (50a, 54, 86) and some also have neutralizing activity (50a, 86, 87).

Another major category of virulence attributes of E. coli are the colonizing factors which enable the ETEC to proliferate to high numbers in the small intestine of host species (37, 83, 107, 121, 153, 154). Many of the colonizing factors are proteinaceous, filamentous surface appendages which extend through the cell envelope of the E. coli (13, 36, 128). They are smaller than and distinct from flagellae and are referred to as fimbriae or pili. Pili mediate adherence of ETEC to
small intestinal epithelium. This adherence overcomes the tendency of the small intestine to purge the ETEC into the cecum and colon. Thus, adherence, mediated by pili, facilitates intensive colonization of the small intestine by ETEC. Fimbriae are antigenic and, if known, may be included in the serotypic identification of *E. coli*. A unified system of naming fimbriae has been proposed (125) and the proposed nomenclature will be noted parenthetically after the first mention in this introduction. Human colonization factors (fimbrial antigens) are called CFA I and CFA II (F2 and F3, respectively). Fimbriae associated with bovine ETEC are called K99 (F5). The K99 fimbriae are also associated with ETEC of lambs and pigs. An additional colonization factor antigen that is often, but not always associated with the K99 antigen is the F41 antigen (118, 119). F41 is also a fimbrial antigen (29) but has not been assigned a new name (125). Other fimbrial antigens frequently associated with ETEC of pigs are K88 (F4) and 987P (F6) (106, 117, 122, 153). The K88 fimbriae are a family of antigenically related proteins which have at least one epitope in common and at least one distinct epitope so they are designated K88ab, K88ac, and K88ad (62, 127).

*Escherichia coli* uncommonly can express more than one fimbrial antigen (14, 143) and can express fimbrial antigens such as Type 1 (F1) pili in addition to those associated with small intestinal colonization. Since the fimbriae are antigenic, they are readily detected by a variety of serologic tests such as agglutination or ELISA. Fimbriae of ETEC are often not expressed as readily in vitro as in vivo. Expression of the fimbriae can undergo phase variation (15, 41), other cell envelope
products may obscure *in vitro* expression of fimbriae (121), and growth conditions can enhance or inhibit expression of fimbriae (30, 46, 49, 57, 63, 76, 77), so failure to detect fimbriae antigenically does not preclude genetic capability of the ETEC to express fimbriae.

The fimbriae associated with ETEC can hemagglutinate erythrocytes from a variety of species (4, 43, 45). The specific hemagglutination profile depends on the fimbrial antigen being tested. Additionally, the fimbrial-mediated hemagglutination is not affected by the presence of mannose (1 per cent, w/v) or related sugars in the suspending medium. This contrasts with hemagglutination mediated by Type 1 fimbriae which is inhibited by the presence of mannose in the suspending medium (140). Both Type 1 and the colonization factor fimbriae can mediate adhesion of *E. coli* to tissue culture cells, isolated intestinal epithelial cells, and mucosal epithelium of isolated gut sections (18, 56, 78, 141). Mannose has a similar effect on *in vitro* adhesion and hemagglutination assays (18, 141).

Another category of factors which enhance colonization of the small intestine by ETEC is the capsular antigens. Capsular antigens are polysaccharides and are indicated in the serotype by the letter "K" (85). True capsules (A-type) were first described by Smith et al. (146, 155, 156). Kauffmann later described three groups of K antigens based on susceptibility to heating (85). A-type capsules are acid resistant and resistant to heating at 100°C for 1 hour, but were susceptible to heating at 120°C for 2 hours. In contrast, L-type of K antigens are destroyed by heating the *E. coli* at 100°C for 1 hour. Finally, B
antigens are differentiated from L-type antigens in that the former are partially destroyed by heating at 100°C but could still adsorb L antibodies from immune serum. Kauffmann considered neither L-type or B-type antigens to be true capsules due to their heat lability (85). Recently, the existence of B-type antigens as entities distinct from cell wall ("O") antigens has been questioned (126). The Ørskovs subsequently showed that the B-type antigens are not separable from the O antigens and that A-type capsules are distinct from the polysaccharides of the cell wall and distinct L-type (polysaccharide) antigens exist on a few strains (124). Further analysis has shown that A-type polysaccharide antigens are mucoid, high molecular weight, and electrophoretically migrate to the anode (80).

Enterotoxigenic E. coli which have the A-type polysaccharide capsule are more virulent than their corresponding acapsular mutants. The former proliferate to higher numbers in the small intestine, regardless of whether they also produce fimbriae (121, 151). Further, the capsule appears to enhance colonization of the mucosal wall versus the lumen (66, 121). Capsular antigen of intensively colonized ETEC has been shown to be closely associated with the intestinal epithelial glycocalyx (20, 21).

The ability of both the fimbriae and the capsules to enhance both colonization and virulence of ETEC has been demonstrated by oral challenge of neonatal pigs and calves with strains of ETEC which lack one, both, or neither antigen (66, 109, 121, 151). Encapsulated, piliated (K99') strains which are manipulated to lack either K99 or a capsular
antigen colonize at slightly lower levels and are slightly less virulent than strains which lack neither antigen (121, 151). In contrast, 987P⁺ ETEC seem to be dependent on capsule to colonize and be virulent in the pig small intestine (121).

Lesions induced by ETEC colonization in the small intestine are usually mild. The most common feature is layers of bacteria closely associated with the epithelium of the lower small intestine (6, 7, 75, 108, 130, 132, 164, 173). Neonatal calves (less than 1 day old) are not only more susceptible to the diarrheic effects of challenge with ETEC (148) but also have more severe lesions resulting from that challenge (6, 130, 132, 164). Although the experimental calves were either colostrum-fed, colostrum-deprived, or gnotobiotic, the same lesion was described (varying in severity): mildly to moderately blunted villi covered with low columnar to cuboidal epithelium in the lower small intestine. Also described were neutrophils in the lumen, erosions or focal necrosis of epithelium associated with fibrinopurulent exudate, and congestion of the lamina propria (6, 7, 130, 132, 164). Also described were caps of neutrophils over the dome epithelium of Peyer's patches (6, 7, 165), and degeneration and attenuation of the dome epithelium (165). Attached layers of ETEC were not associated with the lesions of the domes. These lesions were either mild or not observed in the upper small intestine of similar calves. Tzipori, and others (173), reported similar lesions in a neonatal lamb, but observed no changes in lambs inoculated at 5 to 8 days of age.
Variations from the general description above included villous atrophy in the mid-small intestine but not in the proximal jejunum or distal ileum in one 6-day-old conventionally reared calf inoculated with ETEC (159); segmental intestinal villous atrophy in some but not other conventionally born neonatal pigs inoculated with ETEC (108); lesions in gnotobiotic pigs inoculated with one strain (0101:K?) of ETEC but no lesions in gnotobiotic pigs inoculated with a different strain (0147:K89,K88) (75); and no difference between diarrheic (presumably due to ETEC, and mostly to K88+ strains with various O groups represented) and normal littermates (152). It is likely that the differences could be due to unrecognized concomitant infections in conventional animals, and further, as seen in the gnotobiotic pigs, different strains of ETEC may vary in ability to induce lesions.

Infections with rotavirus. Rotavirus (Reo-like virus) was first characterized and associated with diarrheal disease in calves in the late 1960's (102, 103). However, microscopic lesions of a diarrheal disease of infant mice, EDIM (Epizootic Diarrhea of Infant Mice, now known to be caused by rotavirus), had previously been described and associated with a viral infection (2). Subsequently, rotavirus has been associated with diarrheal disease of many mammals and birds.

Rotavirus is a member of the family Reoviridae and like other members of that family it has a segmented genome of double-stranded RNA (99). It is roughly spherical, non-enveloped, 65 to 75 nm in diameter, and has icosahedral symmetry. The viral core consists of the genome enclosed in protein capsids. Rotavirus particles (as they appear
electron microscopically in negatively stained preparations) may be complete, that is with both an inner and an outer capsids, or incomplete, lacking the outer capsid. Only the complete virions, which have a density of 1.35 g/ml, appear to be infective (12). Furthermore, the proteins of the outer capsid confer type-specificity to rotavirus (11) while the proteins of the inner capsid confer group specificity. The molecular genetic biology of rotavirus has recently been reviewed (42). The genome contains 11 segments of unequal size. Because RNA segments of rotavirus from different species and different isolates within a species may have different electrophoretic migration rates, the electrophoregram of a rotavirus cannot be used to accurately predict or compare virulence, genetic relatedness or serotypic relatedness at this time (19, 38, 42, 48, 51, 142).

Rotavirus isolates tend to be species specific but there are several exceptions. Neonatal pigs seem susceptible to infections due to rotavirus from a variety of species, including humans, calves, and equines (51, 67, 167, 169, 178, 181). There is also variability between isolates from one species in their ability to infect neonatal pigs (169). In contrast to pigs, calves tend to be resistant to challenge with rotavirus isolated from other species (169, 177), but exceptions have been reported (177, 185). Infectious challenge with rotavirus of heterologous species readily stimulates antibody response to the group specific antigens of the inner capsid (51, 167, 169, 177, 181). However, the ability of a rotavirus strain to confer protection against challenge with a second strain appears to depend on a high degree of
antigenic relatedness between the vaccine and challenge strains, especially between antigens of the outer coat proteins which are also associated with neutralizing activity in cell culture (51, 138, 177).

Although rotavirus diarrhea tends to be a disease of neonates (Reviews: 47, 100), older animals may be infected (92, 94, 101, 158, 177, 178). Human rotavirus diarrhea tends to peak at 6 months to 2 years after birth (9, 64, 90, 136), but there are several reports of rotavirus-associated diarrhea in adult humans (25, 69, 98, 137, 174, 175).

The signs and lesions of natural or experimental rotaviral diarrhea are similar regardless of the species examined (67, 81, 82, 96, 102, 103, 131, 133, 157, 166, 170, 173, 178-180, 182). The naturally occurring disease described by Mebus (103) occurred in calves up to 6 weeks old and was characterized by high morbidity, rapid onset, and watery, yellowish feces which, as the disease progressed, contained mucus. Calves became dehydrated and had mild fever. Although some calves were described as moribund, mortality varied from 0 to 50% (102, 103). Similar outcome with high morbidity, low mortality and similar signs has been described for other calf outbreaks (1, 31), in foals (32, 168), infants (72, 137), and pigs (135, 178). In addition, vomiting often precedes the onset of diarrhea in pigs (178). Experimental infections with rotavirus are most often done in specific-pathogen-free or gnotobiotic animals due to the difficulty in preventing spontaneous rotavirus infection in conventionally reared animals and to separate the effects of rotavirus from effects of other organisms.
Lesions in calves are confined principally to the small intestine (7, 102, 115, 133, 159). Grossly, however, the cecum, colon, and rectum may have yellow fluid contents. Similar contents are sometimes seen in the small intestine. Histologically, villi are shortened, broadened, sometimes fused to adjacent villi, and are covered with low columnar, cuboidal or in the most severe cases, squamous epithelium (35, 102, 164). The amount of the villus covered by cuboidal epithelium varies both between calves and between sites along the length of the small intestine (35, 102, 164). The lesions are more severe in calves which are examined later in the course of the disease (35, 102). Both Nebus (102) and Dubourguier (35) described desquamation of epithelium in the most severely affected calves. However, such a lesion must be interpreted with caution as Pearson and Logan (129) have described rapid onset of postmortem changes, especially in infected calves. Viral antigen can be demonstrated in the cytoplasm of villous epithelium. It is more readily demonstrable early than late in the course of disease (102). Cytoplasmic fluorescence of epithelial cells may occur only at or near the tips of villi or it may extend from near the crypt mouth to the villus tip, but usually is confined to the upper half to two-thirds of the villus (102). The fluorescence is rarely continuous, that is, fluorescent patches or single cells may be separated by areas of non-fluorescing cells. Virus-like particles can be detected electron-microscopically within epithelial cytoplasm in a pattern similar to that seen with fluorescence (35, 161). Ultrastructural alterations of epithelial cells include vacuolated cytoplasm, electron dense cytoplasm,
irregular microvillous border with loss of the terminal web, and as noted above, virus-like particles may be seen in the cytoplasm, often within cisternae of endoplasmic reticulum (35, 161). Torres-Medina (165) recently reported mild, focal necrosis of follicle-associated epithelial cells (dome epithelium) which did not affect the gross appearance of the follicle domes. Although virus-like particles could be seen in and near the dome epithelium, the virus appeared to have been phagocytosed rather than replicating (165). The cecum and colon of inoculated calves rarely become infected or have demonstrable lesions (35, 102, 164).

Rotavirus (EDIM - Epidemic Diarrhea of Infant Mice - virus) infections of infant mice differ from those described above in two significant aspects. First, the lesions are milder. Although lesions can occasionally be seen on the lower half of the villus, they are usually confined to the region near the tip and consist of vacuolated, swollen epithelial cells, which have irregular microvilli (2, 24a). The vacuolated cells may contain lipid, viral precursors, viral particles, and swollen, vacuolated mitochondria (2, 24a). Coelho and others (24a) saw tubular structures in the nucleus and cytoplasm of murine enterocytes which were similar to those seen in the nucleus of pig enterocytes by Pearson and McNulty (131). Increased numbers of inflammatory cells were not seen in the lamina propria. Second, rotavirus antigen was readily detected in the colon in addition to the small intestine (96) - a finding which is uncommon in calves and pigs.
Dual infections with *E. coli* and rotavirus  Naturally occurring
dual infections with *E. coli* and rotavirus associated with diarrheal
disease have been reported for humans (8, 40, 162), neonatal and weaned
pigs (5, 92, 116) and calves (1, 68, 103, 107, 115). The incidence of
combined infections with *E. coli* and rotavirus was less than 10 per cent
in all those studies. Identification of combined infections with
rotavirus and ETEC was even lower. Most attributed minimal or uncertain
significance (1, 5, 8, 39, 107, 116, 162) while others reported
significant interaction (92, 103, 115) between the dual infections.

Because of the difficulty in evaluating the significance of natu­
rally occurring dual infections, several groups have experimentally
combined infections with *E. coli* and rotavirus. Host animals included
foals (172), pigs (93, 171), lambs (173, 183, 184), and calves (34, 58,
102, 159, 161, 164, 165, 170). The combined infection with equine
rotavirus and bovine ETEC in foals resulted in diarrhea while mono­
infections did not. However, experimental interpretation of the results
was complicated by additional experimental treatments and varying age of
foals at the time of inoculation with the experimental organisms (172).
More severe diarrhea occurred in weanling pigs with dual infections than
in similar pigs with monoinfections (93, 171). Additionally, high
energy diet fed *ad libitum* (171) or high solids diet fed 3 times a day
(93) intensified the diarrheal disease in weanling pigs. Dual ETEC and
rotavirus infections of lambs were either not more severe than single
infections (173) or were more severe than single infections (183, 184).
The major differences between the two studies were the strains of ETEC
and the experimental animals. Tzipori et al. used an ovine origin ETEC strain (08:K87,K99) and mainly gnotobiotic lambs whereas Wray et al. used a bovine origin ETEC strain (09:K30,K99) in conventionally born, colostrum-deprived lambs.

Most experimental attention has been given to combined *E. coli* and rotavirus infections of calves. Mebus (102) used a nonenterotoxigenic *E. coli* and rotavirus to infect two calves. Both calves had diarrhea earlier than calves infected only with rotavirus but only one developed septicemia with the *E. coli* and became moribund. Mebus characterized the combined infection as significantly more severe than the single infection. He proposed that rotavirus infection in the intestine enhanced or permitted the translocation of an otherwise innocuous *E. coli* across the intestinal wall and subsequently into the non-intestinal tissues. Others have noted *E. coli* infections of nonenteric tissues in animals that had diarrhea associated with rotavirus infections (107, 115, 183).

Other workers have focused primarily on interaction of dual infections with ETEC and rotavirus in calves and have reported variable results. The interactions of the dual infections were described as synergistic (58, 159, 170) or additive (164). The experimental treatments were not consistently repeated in any of the calf models and the severe disease also was not consistently reproduced. Most calves were neonates (1 to 2 days old) that were gnotobiotic, conventionally born but colostrum deprived, or conventionally born and suckled. Although the natural syndrome occurs in 5- to 10-day-old animals, younger animals were used because natural exposure of the calves to the organisms was
presumed to occur in the first few days of life. In contrast, Snodgrass et al. (159) used conventional 5- to 8-day-old calves in an attempt to mimic the naturally occurring disease. He observed more intensive colonization of the small intestine by ETEC in calves infected with rotavirus than in calves not infected with rotavirus. However, the disease produced did not appear to be more severe. He described the interaction as synergistic due to the effect on colonization. The effect of rotavirus infection on ETEC infection remains unclear.

Under natural conditions the health of neonatal animals is influenced by many factors. These include quality of passive immunity obtained prior to or shortly after birth, climatic or environmental stress, genetically mediated resistance or susceptibility, exposure to a variety of organisms (pathogens and non-pathogens), age at the time of exposure, and pathogenicity and virulence of the organisms. This dissertation examines, in part, influence of host age; influence of virulence factors of ETEC; influence of concomitant infections; and influence of environmental factors on concomitant infections as these factors relate to diarrheal disease caused by ETEC in neonatal pigs and calves.
DEVELOPMENT OF RESISTANCE WITH HOST AGE TO ADHESION OF K99+ ESCHERICHIA COLI TO ISOLATED INTESTINAL EPITHELIAL CELLS

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When isolated intestinal epithelial cells from neonatal and older pigs, calves, and mice were tested for adhesion by K99* enterotoxigenic Escherichia coli, cells from older animals were resistant to adhesion.
INTRODUCTION

Neonatal pigs, calves, and lambs are very susceptible to diarrheal disease caused by enterotoxigenic *Escherichia coli* (ETEC), but clinical infections caused by ETEC have not been reported in the adults. Colonization of the small intestine of these species is facilitated by pili (K88, K99, or 987P) which effect adherence to intestinal epithelium (5a). K99⁺ ETEC have been associated with diarrheal disease of newborn pigs, calves, and lambs. Calves and lambs become resistant to experimental challenge with K99⁺ ETEC by 2 days of age (7). The K99⁺ ETEC have not been reported from the diarrheal disease in pigs after weaning at 3 to 8 weeks old. In contrast, K88⁺ ETEC are associated with diarrheal disease of pigs during both the neonatal and postweaning periods (8). Additionally, neonatal mice are susceptible to K88⁺ and K99⁺ ETEC but develop resistance to these strains as adults (4).

The purpose of this study was to determine the adhesion of K99⁺ ETEC to isolated intestinal epithelial cells taken from neonatal and older pigs, calves, and mice.
MATERIALS AND METHODS

Epithelial cell isolation  [See Appendix A for a detailed explanation for our method.] Epithelial cells were isolated from the entire small intestine (3, 9) of pigs 1 day (neonatal), 3 weeks, or 6 weeks old (Fig. 1). Six of nine neonatal pigs and one of three 6-week-old pigs were colostrum deprived and reared on artificial diets in isolation. The remainder was naturally born and reared. Epithelial cells were isolated and pooled from 1-m segments of the upper, middle, and lower thirds of the small intestine of calves 12 hours, 4 days, or 2 weeks old (Fig. 2). The calves were separated from the dams at birth, fed colostrum from a bottle for the first 12 h, and reared in isolation. Epithelial cells were isolated from the entire small intestine of 2-day-old, 2-week-old, or postparturient CFl mice (ARS, Sprague Dawley, Madison, Wis.) (Fig. 3). All epithelial cells were suspended in Mg\(^{2+}\), Ca\(^{2+}\) free phosphate-buffered saline at about 2 \(\times 10^6\) epithelial cells per ml and stored at 4°C until used.

E. coli strains  The E. coli are listed in Table 1. Strains 431 and B41 were grown on Minca agar (minus glucose plus IsoVitaleX), which enhances expression of K99 (2). Strains 263 and 123 were grown on sheep blood agar. Strain 263, which carries the K88 pilus, was used as a strongly adherent control; strain 123, which has neither K99 nor K88 pili but which may express the type 1 pilus, was used as a nonadherent control. After overnight incubation at 37°C, the bacterial cultures were suspended in 0.85% NaCl so the optical density at 430 nm
Table 1. *E. coli* strains with serotype, known pilus type, origin, and enterotoxigenicity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Pilus</th>
<th>Animal of origin</th>
<th>Enterotoxin&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>B41</td>
<td>01:K99:NM</td>
<td>K99</td>
<td>Calf</td>
<td>ST</td>
</tr>
<tr>
<td>431</td>
<td>01:K30,99:NM</td>
<td>K99</td>
<td>Pig</td>
<td>ST</td>
</tr>
<tr>
<td>263</td>
<td>08:K87,88ab:H19</td>
<td>K88</td>
<td>Pig</td>
<td>LT,ST</td>
</tr>
<tr>
<td>123</td>
<td>043:K-:H28</td>
<td>None</td>
<td>Pig</td>
<td>None</td>
</tr>
</tbody>
</table>

<sup>a</sup>ST, Heat-stable enterotoxin; LT, heat-labile enterotoxin.
was 2.2 (about $10^9$ E. coli per ml). This suspension or a fivefold concentration was used in the adhesion tests.

**Adhesion assay** The *in vitro* adhesion assay was conducted as previously reported (3). A trial consisted of cells from a neonate paired with cells from one or two older animals. Each E. coli suspension was individually mixed with an equal volume of each epithelial cell preparation so the ratio was roughly 500 or 2,500 E. coli per epithelial cell, depending on the concentration of E. coli used. Two microscopists counted the number of E. coli adhering (up to 70 E. coli per cell) to each of 20 epithelial cells in a mixture (total of 40 cells per mixture). The host age of the isolated epithelial cells and strain of E. coli in a mixture was unknown to the microscopists. Each trial was repeated at least three times, each on a different day with freshly grown cultures of E. coli. An analysis of variance was used and, when necessary, the sum of the squares was partitioned to make the desired comparisons in each species.
RESULTS

The results exemplified (Fig. 1) were obtained by using neonatal and 6-week-old pigs from three different herds (total of four herds tested). Results with colostrum-deprived and conventional pigs were similar and were combined for statistical analysis. From 8.8 to 14.5 (95% confidence interval) more K99$^+$ ETEC adhered to epithelial cells from the neonates than adhered to the cells from the 6-week-old pigs. Both K99$^+$ strains adhered to epithelial cells from neonatal pigs in significantly greater numbers ($P \leq 0.001$) than strain 123 but in lower numbers than strain 263. The resistance to adhesion by K99$^+$ ETEC, characteristic of cells from 6-week-old pigs, was not demonstrable with cells from 3-week-old pigs. The K99$^+$ ETEC adhered to cells from the 12-hour-old calf in significantly greater numbers ($P \leq 0.001$) than to cells from the 2-week-old calf (Fig. 2). Both K99$^+$ strains adhered in significantly greater numbers ($P \leq 0.001$) to cells from the neonatal mice than to cells from the adult mice (Fig. 3). Strain 263 consistently adhered to epithelial cells of the host species in much greater numbers than did strain 123. Epithelial cells from animals of different ages did not vary in their susceptibility to adhesion by these two strains.
Fig. 1. Number of \textit{E. coli} adherent to intestinal epithelial cells isolated from pigs of different ages. Results with epithelial cells isolated from four pigs (1 day old), two pigs (3 weeks old), and three pigs (6 weeks old) are presented. Bars represent the mean ± one standard deviation of the number of adherent bacteria per epithelial cell. Bacteria were used at 5 \times 10^9/ml and epithelial cells at 2 \times 10^6/ml
E. coli STRAIN & PILUS TYPE
Fig. 2. Number of *E. coli* adherent to intestinal epithelial cells isolated from calves of different ages. Results with epithelial cells isolated from one calf (12 hours old), two calves (4 days old), and one calf (2 weeks old) are presented. Bars represent the mean ± one standard deviation of the number of adherent bacteria per epithelial cell. Bacteria were used at $5 \times 10^7$/ml and epithelial cells at $2 \times 10^6$/ml.
E. coli STRAIN & PILUS TYPE

- 263 K88
- 431 K99
- B41 K99
- 123 TYPE 1?

Legend:
- ■ 12 Hr. OLD
- □ 4 DAY OLD
- □ 2 Wk OLD
Fig. 3. Number of *E. coli* adherent to intestinal epithelial cells isolated from mice of different ages. Results with epithelial cells isolated and pooled from 50 mice (2 days old), 35 mice (2 weeks old), and 10 mice (adult). Bars represent the mean ± one standard deviation of the number of adherent bacteria per epithelial cell. Bacteria were used at $1 \times 10^9$/ml and cells at $2 \times 10^6$/ml.
**E. coli** STRAIN & PILUS TYPE

- **2 DAY OLD**
- **2 Wk. OLD**
- **ADULT**

- **263 K88**
- **431 K99**
- **841 K99**
- **123 TYPE 1?**
DISCUSSION

The data support the hypothesis that epithelium in the small intestine develops resistance to K99-mediated adhesion with increasing host age. The resistance demonstrated seemed to be innate rather than antibody-mediated because: (i) cells of 6-week-old pigs from three herds resisted adhesion by K99+ ETEC but were susceptible to adhesion by K88+ ETEC even though K88 antigen occurs more commonly than K99 antigen among pig ETEC (1, 5b); (ii) cells from a 6-week-old colostrum-deprived pig were resistant to K99 mediated adhesion; (iii) a similar pattern occurred in the three species tested even though K99+ ETEC are not reported from natural diarrheal disease in mice.

ETEC from both neonatal and weanling pigs have been tested for K99 antigen (5a, 5b). To date, K99+ isolates have been confined to ETEC from neonates. The resistance to adhesion reported here may occur in vivo and contribute to this age distribution in pigs. However, cells from 4-day-old calves and 2-week-old mice were susceptible to adhesion by K99+ ETEC. It seems unlikely that the marked resistance to K99+ ETEC which calves develop by 2 days of age and which mice develop before adulthood can be explained by resistance to adhesion as tested in this system.

Effects attributable to the E. coli were also observed. The acapsular strain B41 consistently adhered in greater numbers than the capsular strain 431 which may reflect masking of the K99 pilus by capsular material (6). Even with neonatal cells, the K88+ strains
adhered in greater numbers than the K99⁺ strains. This could reflect more avid adhesive forces, more pili, or more host cell receptors for the K88⁺ strain than for the K99⁺ ETEC. Paradoxically, the K88⁺ strain adhered in very high numbers to the calf cells even though such strains have not been shown to colonize calf intestine in vivo. However, as previously reported for K88⁺ ETEC adherent to calf intestinal epithelial cells in vitro (9), the observed adhesion occurred predominantly on basolateral membranes rather than on brush borders. Adhesion of the K88⁺ and K99⁺ ETEC to other host cells and the K99⁺ to calf cells tended to be random. There was also extensive adhesion between the K88⁺ ETEC.

In conclusion, the variation among ETEC in their host species specificity is generally recognized. Apparently, there is also variation among ETEC in their host age specificity.
LITERATURE CITED


CAPSULE REDUCES ADHERENCE OF ENTEROTOXIGENIC *ESCHERICHIA COLI* TO
ISOLATED INTESTINAL EPITHELIAL CELLS OF PIGS

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ABSTRACT

Previous reports have demonstrated that heat stable (A-type) capsule on piliated enterotoxigenic *Escherichia coli* (ETEC) enhances colonization of ETEC in the small intestine and enhances virulence of ETEC. In this report, four encapsulated ETEC strains and one encapsulated nonenterotoxigenic (NETEC) strain of *E. coli* and their non-encapsulated mutants were tested for adhesion to isolated intestinal epithelial cells or brush borders from neonatal pigs. The ETEC also expressed the K99 pilus antigen. The nonencapsulated mutants of the four ETEC adhered in higher numbers than did the encapsulated parental strains. Both the encapsulated and nonencapsulated forms of ETEC strain 431 grown at 18°C (K99 production suppressed) adhered poorly to the isolated cells. The NETEC strain 1793 which does not express K99 antigen also adhered poorly in both encapsulated and nonencapsulated forms. F\textsubscript{ab} fragments of anticapsular IgG failed to block the effect of capsule on adherence of strain 431. The results indicated that K99 was the principal mediator of *in vitro* adhesion of the ETEC strains and that capsule impedes the *in vitro* adhesion. They also suggested that the capsular enhancement of colonization by such strains *in vivo* probably is by some mechanism other than enhanced adhesion to epithelium.
INTRODUCTION

Colonization (proliferation to high numbers) by enterotoxigenic Escherichia coli (ETEC) in the small intestine of animals contributes to the virulence of ETEC. Several in vivo and in vitro studies have shown that specific filamentous, protein appendages called pili or fimbriae which are on the surface of ETEC facilitate adhesion of ETEC to small intestinal mucosa and facilitate colonization by ETEC in the small intestine (9, 19, 20, 24, 26). Pilius antigens associated with ETEC of pigs are currently named K88, K99, 987P and F41. Ørskov and Ørskov have proposed a pilus antigen nomenclature in which K88, K99, and 987P are called F4, F5, and F6, respectively (21). K99 and F41 are also associated with ETEC of calves and lambs.

Bacterial polysaccharide capsule is associated with bacterial colonization of many inert and biologic surfaces (3). Many strains of ETEC from animals produce capsules (K antigens) of the A-type (heat stable, mucoid, high molecular weight, and low electrophoretic mobility toward the anode) (22). These A-type capsules tend to enhance the colonizing ability of the strains that produce them (7, 20, 26) in that piliated acapsular mutants of many strains colonize the small intestine less intensively than do the encapsulated parental strains. Capsular polysaccharide is closely associated with the ileal microvilli and glycocalyx of calves colonized with encapsulated ETEC (1, 2).

Recent reports indicated that an encapsulated ETEC strain was more virulent in suckling neonatal pigs than was its acapsular mutant and capsular antigen was shown to protect against challenge with the
Conference, in press). Possible mechanisms by which polysaccharide capsule enhances colonization and virulence, suggested by Hunter et al. (3), included mediation of adherence (possibly in conjunction with pili), stabilization of microcolonies, protection from secreted products in the intestinal lumen, protection from secreted products of the immune system, and resistance to phagocytosis.

Adherence of ETEC to isolated membranes, cells or tissue sections of the small intestinal mucosa is an in vitro correlate to an in vivo colonization by ETEC (5, 9, 24). This system has been used to study pilus-mediated adherence of ETEC. In this report, several strains of encapsulated piliated (K99+) E. coli and their acapsular mutants were used to determine if A-type capsule enhances pilus-mediated adherence to isolated intestinal epithelial cells. The data do not support that hypothesis.
MATERIALS AND METHODS

E. coli strains The encapsulated parental strains are listed in Table 1. Acapsular mutants were selected from nonmucoid variants of isolated colonies of the parent strains grown on 5% sheep blood agar for 48 to 72 hours at 37°C unless stated otherwise (27). Acapsular mutant 126 previously had been selected from strain 431 (20). Strains used for the adhesion assays were grown on minca agar (minus glucose plus Isovitalex) which enhances expression of K99 pili (6). The bacteria were suspended from agar in Krebs-Henseleit (KH) buffer, pH 7.4 (24) with 1% (W/V) D-mannose (KHM) added. The KHM suspended cultures were adjusted to about 2 x 10^9 E. coli/ml as determined spectrophotometrically. Actual concentration of viable E. coli was determined using a spiral dilution method (Spiral Systems, Inc., Cincinnati, OH 45244). To study the effect of concentration, bacteria of strain 431 and its acapsular mutant, 126 were varied over a 15-fold range of concentration. Presence of capsule was determined by colonial morphology on agar (27) and agglutination of resuspended cultures in anti-capsule (K-antigen) specific antiserum and failure to agglutinate in anti-cell wall (O-antigen) specific antiserum. Absence of capsule was demonstrated by nonmucoid colonial morphology, agglutination of resuspended culture in cell wall specific antiserum, and failure to agglutinate in capsule specific antiserum. The antisera were obtained from Dr. Richard Wilson (E. coli Reference Center, Pennsylvania State University, State College, PA 16802). All strains were tested for
Table 1. Some characteristics of encapsulated parent strains of *E. coli* used in the *in vitro* adhesion assays

<table>
<thead>
<tr>
<th>Strain #</th>
<th>Serotype</th>
<th>Enterotoxin (STa)</th>
<th>Species of Origin</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>431</td>
<td>0101:K30,99:NM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>Swine</td>
<td>Moon (15, 17)</td>
</tr>
<tr>
<td>1793</td>
<td>0101:K30:H1</td>
<td>0</td>
<td>?&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Moon (16)</td>
</tr>
<tr>
<td>B44</td>
<td>09:K30,99:NM</td>
<td>+</td>
<td>Bovine</td>
<td>Smith (25)</td>
</tr>
<tr>
<td>Troyer</td>
<td>09:K35,99:NM</td>
<td>+</td>
<td>Swine</td>
<td>Kohler (12)</td>
</tr>
<tr>
<td>77-0096</td>
<td>0101:K28,99:NM</td>
<td>+</td>
<td>?</td>
<td>Wilson&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>NM = Nonmotile.

<sup>b</sup>? = Not recorded.

<sup>c</sup>*E. coli* Reference Center, Pennsylvania State University, State College, PA.
expression of K99 antigen by slide agglutination with specific anti-
serum (18).

**Intestinal epithelial cells** Isolated intestinal epithelial
cells (enterocytes) were prepared by a modification of a previously
described method (28) [See also Appendix A]. Suckling pigs 2 to 5 days
old from 3 different herds were sacrificed, and their small intestines
were removed and stored for 1 hour in KH buffer at 4°C. Segments were
inverted onto stainless steel rods (3/16" diameter), shaken at high
frequency, low amplitude in KH buffer for 10 minutes each. The entire
small intestine was used. The resultant cell suspensions were washed 3
times by low speed (200 x g) centrifugation, followed by resuspension
in KHM buffer. The final suspension was adjusted to 2 x 10^6
enterocytes/ml, as determined by counting enterocytes in a hemacy-
tometer chamber. The suspensions were used without further washing.
Fresh enterocytes were isolated each week.

**In vitro adhesion assay** Equal volumes (0.5 ml) of enterocyte
suspension and *E. coli* suspension were mixed, then incubated at 37°C
with shaking for 1 hour. The mixture was washed twice by centrifu-
gation at 200 x g and resuspended in about 1 ml KHM buffer. The final
pellet was suspended in a minimal (about 0.1 ml) amount of KHM. The
suspensions were coded and bacteria adherent to the first twenty
enterocytes or brush borders were counted microscopically, using 1000 x
magnification and differential interference contrast optics, by each of
two observers (40 cells total counted). An upper counting limit of 70
bacteria/enterocyte was set arbitrarily. The results were decoded, and
the mean ± standard deviation was calculated for each 20-cell sample in a replicate. Three such replicate assays were done on consecutive days, using freshly grown bacteria each day.

Preparation of \( F_{\text{ab}} \) fragments

A protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Upssala, Sweden) liquid chromatography column was used according to the supplier's recommendations to isolate IgG from the serum. \( F_{\text{ab}} \) fragments of anti-K30 and anti-K88 antisera were prepared by the method of Mage (13). Undigested IgG and \( F_c \) fragments were removed from the mixture by passing it over the Protein A-Sepharose CL-4B column. The \( F_{\text{ab}} \) solution was concentrated 10-fold with a Minicon B15 concentrator (Amicon, Danvers, MA 01923). After concentration, there was about 1.4 mg protein/ml solution.
RESULTS

Nonencapsulated ETEC consistently adhered to enterocytes in higher numbers than did the respective encapsulated parental strains (Fig. 1). Encapsulated or nonencapsulated forms of strain 431 grown at 18°C adhered poorly to the isolated epithelial cells (Fig. 1). Strain 1793, which is nonenterotoxigenic and does not produce any known ETEC associated pili, adhered poorly in both the encapsulated and nonencapsulated forms.

Anti-K30 F\textsubscript{ab} fragments mixed with strain 431 did not interfere with adherence (Fig. 2). The fragments did not cause agglutination of strain 431 E. coli. The presence of F\textsubscript{ab} on the surface of the strain 431 E. coli was demonstrated by agglutinating the F\textsubscript{ab}-treated E. coli with anti-F\textsubscript{ab} serum. Similarly F\textsubscript{ab}-treated strain 431 E. coli also were agglutinated in anti-K30 serum.

The effect of bacterial concentration on adherence was tested. The results are shown graphically in Fig. 3. The increased adherence with increasing concentration of E. coli is roughly parallel for the encapsulated strain 431 and its acapsular mutant strain 126. At equivalent concentrations, the acapsular mutant adhered about 3 times greater than its encapsulated parent strain.

There was variability (not obvious in Fig. 1) in the number of E. coli adherent to epithelial cells of pigs from different litters, and occasionally of pigs from the same litter. This phenomenon is exemplified in Fig. 4, which depicts the results of an experiment in which epithelial cells from pigs in two litters were compared in one series.
Fig. 1. Nonencapsulated ETEC adhered to isolated enterocytes in higher numbers than did the respective encapsulated parental strains. All strains except 1793 produce K99 pilus antigen (strain 431 grown at 18°C does not express the K99 antigen). The bars represent the mean ± standard error. Triplicate assays were done on the cells from each pig (8 pigs for strain 431, 10 pigs for strains B44 and Troyer, and 6 pigs for strains 77-0096 and 1793)
Fig. 2. There was no effect of anti-K30 $F_{ab}$ fragments on adhesion of strain 431 to isolated intestinal epithelial cells. KHM is Krebs-Henseleit buffer with 1% Mannose. K30 and K88 are the antigens against which the $F_{ab}$ fragments were directed. Anti-K88 $F_{ab}$ was used as a control for non-specific binding. The assays were done in triplicate for each pig. Only KHM was used with the acapsular mutant strain I26. The bars represent the mean ± standard error for triplicate assays.
Fig. 3. There was an effect of concentration on adherence of strain 431 and its acapsular mutant, strain I26. The data are pooled from triplicate assays using enterocytes from four pigs. The standard error of the mean bacterial concentrations shown was $\pm 0.1$ at all concentrations. The T-bars represent the standard error of the triplicate assays. The dashed horizontal line at 70 \textit{E. coli}/epithelial cell indicates the arbitrary upper counting limit.
of adhesion assays. The adherence of both the encapsulated strain 431 and its acapsular mutant 126 was greater to cells of pigs in litter A (pigs 50 and 51) than to cells of pigs in litter B (pigs 52 and 53).
Fig. 4. There was variation in the number of bacteria adherent to epithelial cells of pigs from different sources. Litters A and B were from different herds, and both litters had suckled unvaccinated dams. The bars represent the mean ± standard error of triplicate assays using 2 pigs per litter.
E. coli / EPITHELIAL CELL

LITTER A  LITTER B  LITTER A  LITTER B

431  126
DISCUSSION

The data presented did not support the hypothesis that polysaccharide capsule (A-type) enhanced pilus-mediated adhesion of ETEC to isolated intestinal epithelial cells. We previously observed that a nonencapsulated strain of E. coli adhered better in vitro than did an encapsulated strain in a system similar to that used here (23). However, the strains had been isolated from different species, which limited the significance of the observation. In this study, four ETEC strains were selected on the basis of capsular antigen, cell wall antigen, and K99 pilus antigen. All four strains, representing three different A-type capsular antigens, behaved similarly in the adhesion assays; that is, the acapsular mutant adhered in higher numbers than did the encapsulated parent strain. A previous report indicated that the K103 capsule of a 987P piliated E. coli did not enhance in vitro adhesion to porcine epithelial cells (9). In contrast to the results with K99+ ETEC reported here, there was no evidence that capsule reduced in vitro adherence of 987P+ ETEC (9). The 987P pili are structurally, chemically, and antigenically distinct from K99 pili (4, 8, 10, 11), and are likely to interact differently with surrounding capsule and epithelial cells.

Anti-K30 F\textsubscript{ab} did not affect adherence of the encapsulated 431 (K30+) ETEC. The failure of anti-K30 F\textsubscript{ab} to block adhesion suggests that K30 is not mediating adhesion and is consistent with observations that anti-pilus F\textsubscript{ab} inhibited adherence of homologously piliated E. coli, while F\textsubscript{ab} against non-pilus antigens had no effect on adhesion.
(9) If capsule sterically hindered the adherence, addition of $F_{ab}$ fragments would have been expected to further contribute to hindrance or possibly to have no additional effect. It is possible that in this case too little anti-K30 $F_{ab}$ was available either to block or contribute to the effect of capsule, since *E. coli* coated with the anti-K30 $F_{ab}$ still was agglutinated with anti-K30 serum.

One possible explanation for the reduced adherence of the encapsulated strain 431 is that the space occupied by the capsule nonspecifically blocked cell receptors which would otherwise be exposed for attachment by piliated bacteria. That does not seem to be true, since varying the concentration of strain 431 *E. coli* (Fig. 3) used in the assay showed that about 2-fold more bacteria adhered at the highest concentration than at the concentration routinely used. This indicated that the brush borders were not saturated with encapsulated *E. coli* at the lower concentration. In contrast, the numbers of adherent bacteria of the acapsular mutant 126 were near the upper counting limit for the assay at concentrations routinely used. It seems likely that *in vitro* the capsule could mask many of the K99 pili on piliated, encapsulated strains, much as it masks the O antigen during serologic characterization. This is supported by the observation of slow, diffuse agglutination of encapsulated, piliated strains in anti-K99 serum compared to rapid, flocculent agglutination of nonencapsulated, piliated mutants. Furthermore, electron micrographs have shown that capsule and pili occur in similar regions over the bacterial cell surface (11).
Two possible sources of variation in adherence which occurred between litters (exemplified in Fig. 4) are differences in passively acquired immunity and differences in congenital or genetic resistance to K99-mediated adherence. The enterocytes were from suckling pigs whose dams had not been vaccinated against *E. coli*. However, their immunity to ETEC is unknown. *In vitro* adherence of piliated *E. coli* can be inhibited by adding specific antipilus antiserum to the *E. coli* suspension (5, 9). While passive immunity to pilus antigen inhibits the effects of *in vivo* challenge with piliated ETEC (14), the effect of passive immunity of the source pigs on *in vitro* adherence of ETEC has not been reported. In contrast, genetic resistance of pigs to K88+ ETEC is apparent as both lack of response to *in vivo* challenge and as decreased adherence *in vitro* (24). If that phenomenon occurs with K99+ ETEC, the effect is not as marked as it is with K88+ ETEC. There is variation among neonatal pigs in their response to challenge with ETEC strain 431. In one study, 50% of the challenged pigs were resistant to lethal effects of strain 431 (14) and more recently, only 26% were resistant to similar challenge (16). The observations presented here are consistent with, but do not prove, congenital or genetic resistance to K99-mediated adherence.

The data in this report suggest that the mode by which capsule enhances colonization is not through enhanced adherence to intestinal mucosa. While the *in vitro* adhesion assay correlates with *in vivo* colonization in many cases, it is an artificial system isolated from normal physiologic effects, such as gut motility, the digestive milieu,
and secretory and cellular immune processes. Furthermore, *in vitro* adhesion which is assayed after 1 hour of incubation is likely to represent a small fraction of the events occurring during ETEC colonization of the small intestine, which is usually assayed 18 to 24 hours after oral inoculation. Efforts to define the mechanisms of adherence as they relate to colonization and the roles of capsules in those events will be made using *in vivo* models.
LITERATURE CITED


INTERACTION OF ROTAVIRUS AND ENTEROTOXIGENIC
ESCHERICHIA COLI (ETEC) IN GNOTOBIOTIC CALVES

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ABSTRACT

Five- to eight-day-old gnotobiotic calves were orally inoculated with either the Nebraska strain of Rotavirus, the B44 strain of enterotoxigenic Escherichia coli, or both agents simultaneously. Uninoculated gnotobiotic calves were also included. None of the calves died during the 48-hour period between inoculation and necropsy. Rotavirus and E. coli each produced mild transient diarrhea. The small intestine of the rotavirus inoculated calves had villous atrophy, especially in the upper small intestine. Most calves inoculated with E. coli had slight cuboidal metaplasia of the ileum and moderate neutrophilic infiltration into the villous lamina propria and epithelium. One calf inoculated with E. coli had moderate villous atrophy in the ileum. The diarrheal disease produced by simultaneous inoculations of both agents was somewhat more severe than inoculation of either alone. There was greater frequency and duration of fever, greater frequency and duration of diarrheic feces, villous atrophy at all levels of the small intestine, greater colonization of E. coli in the ileum, and ulcers in the cecum of two of the dually infected calves. The differences were considered to be the result of additive rather than synergistic interactions. These results were compared to those studies which implied synergistic interactions between E. coli and rotavirus infections in gnotobiotic calves. Important factors of difference which were considered were sequence of inoculation, the timing of necropsy following inoculation, dose of the inocula, age at inoculation, and volume and quality of feed.
INTRODUCTION

Rotavirus (1, 4, 13, 16, 17) and enterotoxigenic E. coli (ETEC) (1, 16, 17, 21, 22) are frequently associated with fatal diarrhea of young calves. Rotavirus induced calf diarrhea is characterized by: an incubation period of 12 to 72 hours; transient, mucoid to watery, yellow diarrheic feces; transient, mild hyperkalemia; and high concentration of rotavirus in the feces (7, 10, 13, 23). Intestinal pathologic changes due to rotavirus typically includes infection and sloughing of villous epithelium into the intestinal lumen, cuboidal to squamous metaplasia of the remaining epithelium, fusion of villi, decrease in villous height (villous atrophy), hyperplasia of crypt epithelium and mononuclear cell infiltration of the villous lamina propria (5, 10, 12). The diarrhea is probably a result of malabsorption and maldigestion (8, 15).

In contrast, inoculation of calves less than one day old with ETEC causes severe, watery diarrhea, weakness, and dehydration with an incubation period of 6 to 22 hours (3, 22). A prominent feature of ETEC is their ability to colonize the small intestine in high numbers (22) and to adhere to intestinal epithelial cells, a feature which may be recognized histologically or with specific fluorescent antibody staining (3, 16, 18). Some ETEC may also cause lesions in the lower small intestine. The lesions include villous atrophy and neutrophilic exudate in the lamina propria, villous epithelium, and sometimes on the epithelium over lymphatic domes of Peyer's patches (3, 18).
There have also been reports of naturally occurring mixed infections of rotavirus and E. coli in calves (1, 2, 8, 16, 17, 19). The significance of the presence of both rotavirus and E. coli in diarrheic calves is equivocal. Of the reports noted, two presented some evidence of increased severity of disease (16, 17), two failed to detect any significant interaction (1, 19), and two had insufficient data to allow any conclusion to be drawn (2, 8).

Neonatal gnotobiotic calves experimentally infected with both rotavirus and E. coli had more severe signs than similar calves infected with either agent alone (7, 12). In one study (12), the interaction between the rotavirus and E. coli, using a strain of E. coli which was non-enteropathogenic by itself, apparently caused one of two calves to become bacteremic with the E. coli strain and promoted a more rapid onset of diarrheal disease than with rotavirus alone. The lesions in the small intestine were not more severe with dual infections than with rotavirus alone with the exception of the presence of greater villous atrophy in the ileum. The results in that study are difficult to interpret since several calves were also contaminated with extraneous bacteria. The interaction of the E. coli and rotavirus was characterized as significant (12). On the other hand, Gouet et al. (7) used ETEC and rotavirus to study interactions in gnotobiotic calves. The mixed infection produced severe diarrhea, dehydration, and death in calves inoculated at less than one day of age, whereas single infections of either of the two agents in similar doses in calves of
the same age did not result in death (7). The interaction of the ETEC and rotavirus was characterized as synergistic (7).

The present study was undertaken to further examine the interaction of an ETEC and rotavirus in the production of diarrhea in gnotobiotic calves.
MATERIALS AND METHODS

Animals The technique used to obtain gnotobiotic calves was similar to one described by Miniats and Jol (14) for delivery of gnotobiotic pigs. Jersey cows in the 274th day of gestation were anesthetized with halothane (Ayerst Laboratories Incorporated, New York, NY 10017) and the calves were delivered by caesarian section. The calves were immediately passed into the isolation chamber through a germicidal trap containing a 10% Wescodyne (West Chemical Products, Inc., New York, NY 11101) solution in water. Once inside the isolator, calves were dried off and their respiration was stimulated by rubbing and by intravenous injection of 1 ml of doxapram hydrochloride (A. H. Robins Co., Richmond, VA 23220). Twice daily, the calves were fed 360 ml of Carnation Evaporated Milk (Carnation Company, Los Angeles, CA 90036) mixed with an equal volume of water. No other nutrients were provided.

Inocula The rotavirus inoculum pool was obtained by orally inoculating calf No. 1 with filtered fecal material from a gnotobiotic calf identified as 76-23 which contained the Nebraska Rotavirus (12) and was donated by Dr. Charles Mebus. The small intestine of calf No. 1 was removed 49 hours after inoculation, combined with an equal mass of 0.85% sterile saline and homogenized. That calf's fecal material which contained rotavirus was collected, diluted with an equal volume of 0.85% sterile saline and was mixed with the intestinal homogenate. The mixture was subjected to two cycles of freezing at 

-70°C and thawing at 37°C. After the second cycle, the mixture was
centrifuged at 11,000 x g, the supernatant fluid was decanted and
centrifuged again at 11,000 x g. The supernatant from that centri-
fugation was passed through a series of millipore filters down to
0.45 \( \mu \)m pore size. The filtrate from the 0.45 \( \mu \)m millipore filter was
diluted in an equal volume of 0.85% sterile saline and filtered through
a 0.22 \( \mu \)m pore size filter. The 0.22 \( \mu \)m filtrate was divided into
10 ml aliquots and was frozen and stored at -70°C as the rotavirus
inoculum pool. Electron microscopic examination of the filtrate showed
characteristic rotavirus particles (13).

The ETEC strain B44 (09:K30,99:NM) (22) was grown overnight in one
liter of trypticase-soy broth (TSB) at 37°C on an oscillating shaker,
centrifuged, and resuspended in 20% glycerin in 0.85% sterile saline to
about 10^9 E. coli/ml. The suspension was divided into 12 ml portions
which were stored at -70°C.

The standard rotavirus inoculum was 10 ml of freshly thawed
material from the rotavirus inoculum pool while the standard B44
inoculum was 1 ml of freshly thawed material. Counts of colony forming
units (CFU) of the B44 inoculum were done on 5% sheep-blood agar plates
(BAP) at the time each calf was inoculated [mean \( \pm \) s.d = 3.3 \( \pm \)
1.5 \( \times \) 10^9].

Gnotobiotic monitoring Fecal samples taken from the rectum
were examined for aerobic and anaerobic bacterial contamination by the
animal supply unit at NADC. Specifically, fecal swabs for aerobic
culture were immediately placed into TSB and incubated at 37°C over-
night. The swabs were streaked onto 5% sheep blood agar plates which
were incubated at 37°C for at least 48 hours. Fecal swabs for anaerobic culture were immediately placed into fluid thioglycollate media and then transferred under CO₂ to prereduced peptone yeast extract broth which was then incubated at 37°C. Aerobic or anaerobic colonies were isolated and identified biochemically. Additionally, a separate group at NADC inoculated fecal and blood samples into TSB and thioglycollate broth which were then incubated at 37°C for at least 48 hours to detect presence of the B44 E. coli strain or other contaminants.

Fecal samples taken at the time of inoculation and at 8-hour intervals thereafter were examined by negative staining in the electron microscope for the presence of virus (13). Pieces of ileum, omasum, kidney, spleen, adrenal gland, and lung were examined for bovine virus diarrhea (BVD) virus for three blind passages on bovine turbinate cells by Dr. A. W. McClurkin at NADC, Ames, Iowa (11). Each passage was checked for cytopathic effects and, if negative, was checked for interfering virus by inoculating the cells with a cytopathic virus. Serum from blood taken at necropsy was tested for neutralizing antibody to BVD, infectious bovine rhinotracheitis (IBR) and parainfluenza 3 (PI-3) viruses (20).

**Clinical observations**  
Clinical signs of disease such as diarrhea, dehydration, strength, attitude, and appetite were evaluated each time the calf was fed and 8-hour intervals after inoculation.

Objective signs such as hematocrit, total protein, and percent fecal dry weight (FDW) were assessed using portions of the same samples
used for gnotobiotic monitoring. Fecal samples with FDW < 15% were considered to be diarrheic. Total fecal mass was not measured.

Intestinal absorption was evaluated by feeding 500 ml of sterile 5% D-xylose in water 24 hours before and after inoculation. The amount of D-xylose was determined in plasma samples taken immediately before the 1 and 2 hour after feeding the D-xylose (24).

Intestinal transit was evaluated by feeding, 1 hour before necropsy, 5 ml of saturated Evan's blue dye in water mixed with 50 ml of milk in place of the regular morning feeding.

**Necropsy and histopathologic examination** Calves were anesthe-
tized with sodium pentobarbital (Abbott laboratories, North Chicago, IL 60064) while in the isolator, removed and placed in lateral recumbency. The peritoneal cavity was exposed and portions of liver and mesenteric lymph node were removed for bacteriologic examination. In order, the four sites sampled in the small intestine were: ileum, approximately 1 m cranial to the ileocecal junction; duodenum; jejunum, approximately 1 m caudal to the duodenum; and midgut, halfway between the ileal and jejunal sections. At each of the sites samples taken were: a 10 cm ligated section for bacterial enumeration and identification; two opened sections pinned to cardboard and immersed in 10% buffered formalin for histologic evaluation; one section, frozen in supporting media in a dry-ice/alcohol bath for frozen sectioning. Additionally, sections from mid-cecum, apex of the spiral colon, omasum, and rumen were taken for histologic evaluation and frozen sectioning. Other tissues which were sampled for histologic evaluation were fundic and
pyloric abomasum, spleen, mesenteric lymph node, liver, kidney, adrenal gland, thymus, lung, and left ventricular wall. The mean villous height and crypt depth was determined for each site in the small intestine by using an ocular micrometer to measure ten villi and ten crypts which appeared to be cut their full length or depth.

Fluorescent antibody techniques were used to detect K99 or rota-virus antigens in the frozen sections (16).

Experimental design Table 1 shows all calves and the inocula used. At least 4 calves in each group were treated in a similar manner, with the exception of one calf which was fed 10 ml of the B44 suspension. The inocula were mixed with the regular morning feeding when the calves were 5 to 8 days old. Necropsy was performed 48 to 50 hours after inoculation.
Table 1. Gnotobiotic calf number, sex, inoculum used, and contaminating agents isolated

<table>
<thead>
<tr>
<th>Group</th>
<th>Calf no.</th>
<th>Sex</th>
<th>Inoculum</th>
<th>Contaminant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>F</td>
<td>None</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>M</td>
<td>None</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>F</td>
<td>None</td>
<td>Bacillus pumilis</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>M</td>
<td>None</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>M</td>
<td>None</td>
<td>---</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>1(^{c})</td>
<td>F</td>
<td>Rotavirus, 76-23</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>F</td>
<td>A</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>F</td>
<td>A</td>
<td>Micrococcus luteus and Erwinia herbicola</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>F</td>
<td>A</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>M</td>
<td>A</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>F</td>
<td>A</td>
<td>---</td>
</tr>
<tr>
<td>B44</td>
<td>2</td>
<td>F</td>
<td>B(^{e})</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>F</td>
<td>B</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>M</td>
<td>10XB</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>F</td>
<td>B</td>
<td>---</td>
</tr>
<tr>
<td>Dually Infected</td>
<td>8</td>
<td>M</td>
<td>A+B</td>
<td>---</td>
</tr>
<tr>
<td>(B44+Rotavirus)</td>
<td>10</td>
<td>F</td>
<td>A+B</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>M</td>
<td>A+B</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>F</td>
<td>A+B</td>
<td>---</td>
</tr>
</tbody>
</table>

\(^a\)F = Female; M = Male.

\(^b\)None detected.

\(^c\)This calf was used primarily to expand the rotavirus pool. Data not included in figure or other tables.

\(^d\)A = 10 ml freshly thawed .22 \(\mu\)m filtered fecal and intestinal homogenate from Calf No. 1.

\(^e\)B = 1 ml freshly thawed B44 E. coli suspension.
RESULTS

**Gnotobiotic monitoring**  BVD was not isolated from the tissues nor were neutralizing antibody titers to BVD, IBR, or PI-3 detected in the serum from any calf. Bacterial contaminants are noted in Table 1. In addition, an unidentified aerobic micrococcus was isolated from two fecal samples from calf No. 12, and *Staphylococcus epidermidis* was isolated from ligated segments of intestine of calf No. 7 and was believed to have been a contaminant at or after necropsy. Extraneous bacteria were not isolated from any inoculum fed to the calves.

**Clinical observations**  Attitude, appetite, strength and state of hydration were normal in the control calves. The inoculated calves ate all the milk offered but nursed less vigorously at the onset of diarrhea (Table 2). With one exception the inoculated calves which developed diarrhea became weak and lethargic 24 to 40 hours after inoculation. One calf in the rotavirus group remained strong and alert throughout the experimental period. Three calves in the dually infected group remained weak but were alert at the time of necropsy while all other calves were strong and alert at that time. Two calves in the *E. coli* group developed transient mild dehydration 24 to 32 hours after inoculation. Three calves in the dually infected group became mildly dehydrated 24 to 32 hours after inoculation and remained so at the time of necropsy.

Some calves in all groups had FDW \(\leq 15\%\) (Table 2). The incidence of fecal samples with FDW \(\leq 15\%\) was greatest in the dually infected group and roughly equivalent among the other three groups (Table 2).
Table 2. Incidence, degree, and onset of fecal dry weights (FDW) ≤ 15% [See Appendix C]

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>% FDW</th>
<th>Onset</th>
<th>K, First fecal sample with FDW ≤ 15%: Mean (range) hours after inoculation.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>affected calves(^a)/ total calves</td>
<td>affected samples/ total samples</td>
<td>Onset(^b)</td>
<td>affected samples</td>
</tr>
<tr>
<td>Control</td>
<td>3/5</td>
<td>10/29</td>
<td>29 (8-48)</td>
<td>11.3 (8.9-13.2)</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>5/5</td>
<td>8/30</td>
<td>21 (8-32)</td>
<td>9.7 (4.4-14.9)</td>
</tr>
<tr>
<td>B44</td>
<td>3/4</td>
<td>8/21</td>
<td>13 (8-24)</td>
<td>8.1 (2.6-13.8)</td>
</tr>
<tr>
<td>Rotavirus + B44</td>
<td>4/4</td>
<td>17/24</td>
<td>18 (8-48)</td>
<td>8.5 (4.7-14.7)</td>
</tr>
<tr>
<td>All calves prior to inoculation</td>
<td>7/18</td>
<td>7/143</td>
<td>---</td>
<td>12.7 (9.9-14.2)</td>
</tr>
</tbody>
</table>

\(^a\)Affected calves were those with fecal dry weights (FDW) ≤ 15% for any sample taken after inoculation.

\(^b\)First fecal sample with FDW ≤ 15%: Mean (range) hours after inoculation.
Of samples with FDW ≤ 15%, the mean dry weight was similar among the inoculated groups (Table 2). The febrile response was most severe among calves in the dually infected group (Table 3). Two calves in each of the rotavirus and dually infected groups developed impaired absorption of xylose. There were no significant changes in the hematocrit or total protein levels in any of the calves following inoculation. The transit of Evans' blue dye through the intestine was slightly less in the control calves than among the inoculated calves and was similar among the three groups of inoculated calves.

**Bacteriologic examination of tissues** Colonization of the small intestine by B44 increased from the duodenum to the ileum. Within the groups infected with B44 only and rotavirus plus B44, colonization was similar at each of the upper three sites, but was much more marked in the ileum of dually infected calves (Table 4). In those calves which were contaminated, the contaminating bacteria were present at $10^2$ to $10^5$ CFU/10 cm segment. *E. coli* strain B44 was isolated from the liver and mesenteric lymph node of calf No. 2 and mesenteric lymph node and peripheral blood of calf No. 16, both of which had been inoculated with B44 alone, and from the mesenteric lymph node of calf Nos. 8, 10, and 21 which had been inoculated with both rotavirus and B44.

**Viral shedding** Initial shedding of rotavirus occurred 24 to 40 hours (mean=32 hours) after inoculation in the monoinfected rotavirus group, while it occurred at 24 to 32 hours (mean=28 hours) in the dually infected group. Shedding continued until necropsy. No calves in the B44 or control groups shed rotavirus at any time.
Table 3. Incidence and degree of fever\textsuperscript{a}

<table>
<thead>
<tr>
<th>Group</th>
<th>affected calves/ total calves</th>
<th>febrile readings/ total readings</th>
<th>Mean ± standard deviation of febrile readings\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0/5</td>
<td>0/29</td>
<td>---</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>1/5</td>
<td>1/30</td>
<td>(39.6 ± 0.0)</td>
</tr>
<tr>
<td>B44</td>
<td>1/4</td>
<td>2/24</td>
<td>(40.1 ± 0.1)</td>
</tr>
<tr>
<td>Rotavirus + B44</td>
<td>3/4</td>
<td>5/24</td>
<td>(40.3 ± 0.6)</td>
</tr>
<tr>
<td>All calves prior to inoculation</td>
<td>0/18</td>
<td>0/220</td>
<td>---</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Affected calves or febrile readings were those which showed a temperature ≥ 1\textdegree C above the temperature just prior to inoculation.

\textsuperscript{b}Fahrenheit degrees were published. Celsius degrees are in parentheses.
Table 4. Colony forming units of B44 in intestinal segments of calves inoculated with B44 only or rotavirus plus B44

<table>
<thead>
<tr>
<th></th>
<th>B44 only</th>
<th>Rotavirus + B44</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>$(2.8 \pm 2.8) \times 10^5$</td>
<td>$(2.6 \pm 4.6) \times 10^6$</td>
</tr>
<tr>
<td>Jejunum</td>
<td>$(1.9 \pm 3.1) \times 10^6$</td>
<td>$(1.8 \pm 1.6) \times 10^5$</td>
</tr>
<tr>
<td>Midgut</td>
<td>$(1.7 \pm 2.5) \times 10^7$</td>
<td>$(4.3 \pm 6.5) \times 10^7$</td>
</tr>
<tr>
<td>Ileum</td>
<td>$(2.0 \pm 1.3) \times 10^7$</td>
<td>$(1.7 \pm 1.8) \times 10^9$</td>
</tr>
</tbody>
</table>

* (Mean ± standard deviation); 4 calves in each group.
Necropsy and histologic examination

The small intestine of control calves had long villi (Fig. 1) with intact epithelium. The mucosa of the cecum and spiral colon tended to be villous. No significant lesions were seen in this group.

There was mild focal cuboidal metaplasia of the ileal epithelium and mild congestion of the ileal lamina propria of the B44 infected group. Villous atrophy was not a feature of calves in this group (Fig. 1), except in calf No. 16 which had moderate villous atrophy, severe congestion of the lamina propria and proteinaceous exudate in spaces where epithelium was separating from the lamina propria near the tips of the villi. The lamina propria, villous epithelium, and reticulo-endothelial tissues had increased neutrophilia. Rarely, bacteria could be seen adhering singly or in small groups to ileal epithelium.

Villous atrophy of the upper small intestinal sites was a feature common to the rotavirus infected calves (Fig. 1). The severity of the villous atrophy decreased from the duodenum to the ileum. The crypts did not appear to be hyperplastic, although incompletely fused villi occasionally gave the impression of elongated crypts.

Villous atrophy in the small intestine of the dually infected calves was of comparable severity to that of the rotavirus group. However, villous atrophy was as severe in the ileum as the other three sites (Fig. 1). In contrast to the B44 group, in the dually infected group bacteria were adherent to the ileal epithelium in layers usually from the crypt mouth to the villous tip. Adherent bacteria were
Fig. 1. Height of villi and depth of crypts. A bar represents the mean with one standard error for each intestinal site for 4 calves in each inoculum group.
present at the midgut site of one calf. Neutrophilia similar to that seen in the B44 group was also seen in the dually infected group. Small, confluent reddened foci were present in the cecal mucosa of two dually infected calves. Histologically these areas were congested and ulcerated. Thrombi were present in mucosal and submucosal vessels. Edema and neutrophilic infiltrate extended from the subserosa to the mucosa.

The eosinophilia in intestinal sections noted by others (10) was also noted here in all calves. Additionally, a diffuse eosinophilia was consistently noted in the thymus, and irregularly noted in the mesenteric lymph nodes and abomasum in the lamina propria at the base of the gastric pits. All calves had minimal mesenteric fat.

Some changes occurred sporadically or could not be associated with a particular infectious agent. Vacuolation and neutrophilic infiltration of squamous epithelium (pustular epitheliitis) of the rumen were seen in one dually infected and one rotavirus infected calf. Crypt abscesses in the small intestine were seen in a control calf and two B44 infected calves. Focal petechial hemorrhages were seen in the colon of a B44 infected calf.

The only significant extra-intestinal lesion seen was a lobular pneumonia in the ventral portion of the right cranial lung lobe of calf No. 12. The pneumonia was characterized by atelectasis, fibrino-purulent alveolar exudate, purulent bronchiolar plugs, capillary congestion, and occasional small rod-shaped bodies. A portion of the affected lung was cultured but no bacteria were isolated.
Fluorescent antibody examination Strain B44 was detected only in those calves inoculated with B44. Rotavirus was detected only in those calves inoculated with rotavirus (Table 5). Rotavirus antigen was consistently present in the duodenal and jejunal sections of infected calves but was not present in midgut or ileal sections of some calves in the dually infected and rotavirus infected groups. Infected cells were usually isolated, occasionally were in groups, and rarely formed sheets covering the upper portion of villi. The B44 adhering to villous epithelium were single or in small groups when detected in the upper small intestine. The B44 adhered in single or multiple layers to the villous epithelium of the ileum.
Table 5. Detection of rotavirus or strain B44 in fluorescent antibody tissue sections

<table>
<thead>
<tr>
<th>Group</th>
<th>Location</th>
<th>K99</th>
<th>Rotavirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Duodenum</td>
<td>0/5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Jejunum</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>Duodenum</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Jejunum</td>
<td>0/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
<td>0/5</td>
<td>3/5</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>0/5</td>
<td>3/5</td>
</tr>
<tr>
<td>B44</td>
<td>Duodenum</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Jejunum</td>
<td>2/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
<td>2/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>2/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Rotavirus + B44</td>
<td>Duodenum</td>
<td>2/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>Jejunum</td>
<td>2/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>Midgut</td>
<td>3/4</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>Ileum</td>
<td>4/4</td>
<td>3/4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of calves positive/total calves.
DISCUSSION

The signs and lesions induced in calves infected with both rotavirus and B44 tended to be more severe than the disease and lesions caused by either agent alone. The calves with dual infection were more frequently diarrheic and had higher and more persistent fevers than monoinfected calves. Ulcers were present in the cecum of two of the dually infected calves but not in the monoinfected calves. Villous atrophy tended to be present in all levels of the small intestine in the dually infected calves but was absent from the ileum of rotavirus calves and was not seen at any level of the B44 infected calves. Colonization of the ileum by strain B44 was greater in dually infected calves than monoinfected calves. In summarizing the interaction between the rotavirus and *E. coli*, the criterion for synergy which we used was that the effect of two or more factors must be greater than the additive effects of those factors considered separately. Despite other differences noted above, the only synergistic effect demonstrated was increased ileal colonization by strain B44 in dually infected calves. The lesions in the intestine of the dually infected calves tended to be no greater than the sum of the lesions which B44 and rotavirus produced independently. The subjective clinical status of the dually infected calves and the monoinfected calves was similar. Finally, none of the calves were near death at the time of necropsy.

Our results must be compared to the data of others who produced a synergistic (7) or significant interaction (12) between *E. coli* and rotavirus. However, comparisons are difficult due to differences in
methodology employed in the studies. Conditions which differ between the current study and those previously reported are characteristics of the inocula, age of calves at time of inoculation, sequence of inoculation, time of necropsy, and volume and quality of feed.

**Inocula**  
E. coli strain B44 is a well characterized enterotoxigenic, enteropathogen of calves (22) which has been associated with villous stunting, cuboidal metaplasia and loss of villous epithelium, and migration of neutrophils through the intestinal epithelium of colostrum-fed conventional calves with fatal infections (3). The appearance of these lesions was variable among the calves in the monoinfected B44 group. When Gouet et al. (7) inoculated gnotobiotic calves with ETEC in doses similar to those used here, the resulting disease was similar to that reported here. In contrast to the B44 used in this study, the strain of E. coli used by Mebus et al. (12) was non-enteropathogenic by itself in gnotobiotic calves. Our use of ETEC strain B44 may have contributed to the differences between our study and that reported by Mebus.

Gouet et al. (7) used a rotavirus strain which had been isolated from a neonatal calf in France. The diarrheal disease which that strain induced in monoinfected gnotobiotic calves was similar to the disease reported here. The rotavirus inoculum used in this study was a gnotobiotic calf-passaged descendent of those originally reported by Mebus et al. to produce significant interaction (13). The small intestinal lesions and mild, transient diarrhea produced in our gnotobiotic calves are similar to those previously attributed to that strain
(13) and other calf rotavirus strains (10, 23). The incubation period reported here is about 10 hours longer than that reported by Mebus et al. (12). This may be due to a lower titer of the inoculum due to the dilutions and multiple filtration of the initial pool, but no quantitative comparisons can be made at this time.

The disease and lesions produced by the ETEC strain B44 and rotavirus in this study were similar to those produced by similar inocula in other studies. It seems unlikely that the inocula we used accounts for the differences between our study and other studies. However, the dose of the inocula may be important.

**Age and sequence of inoculation** The calves in this study were 5 to 8 days old when inoculated simultaneously with B44 and rotavirus. Mebus et al. (12) inoculated calves with *E. coli* early in the second day of life and 24 hours later inoculated rotavirus. Gouet et al. (7), on the other hand, inoculated rotavirus 2 to 3 hours after the calf was born, and 24 hours after birth inoculated *E. coli*. In both studies, severe disease resulted. Therefore, it seems that sequence of inoculation may be of less importance than the calf's age at time of inoculation in producing a synergistic interaction.

**Time of necropsy** The protocol followed may have caused us to miss aspects of interaction which depended on the variable incubation time of the agents used. Both Gouet et al. (7) and Mebus et al. (12) keyed necropsies to clinical signs. Our necropsies were done 2 days after inoculation without regard to clinical signs which were frequently more severe prior to necropsy. The D-xylose absorption test is
considered to be an accurate indicator of intestinal malabsorption in calves (24). Since lesions consistent with malabsorption were consistently present at necropsy of calves in both the rotavirus only and the dually infected groups, the inconsistent results of the D-xylose tests reported here may reflect performance of the tests before some calves become malabsorptive. Alternatively, in spite of intestinal lesions, the remaining epithelium may have absorbed the D-xylose in normal levels. The timing of our necropsies does not allow a distinction to be made between the two possibilities.

**Feed** The amount of feed ingested is an important factor in the pathogenesis of diarrheal disease due to malabsorption (9, 15). Feeding milk *ad libitum* to young dairy calves can result in diarrhea whether the calf had been previously normal or diarrheic (6). Both Gouet et al. (7) and Mebus et al. (12) fed more milk to the calves than we did, but neither group fed mild *ad libitum*. Additionally, the lean condition of our calves at necropsy was suggestive of limited feeding. It is possible that the limited feed in our study prevented severe clinical disease in the rotavirus only or dually infected calves.

In summary, dual infections with rotavirus and *E. coli* strain B44 resulted in diarrheal disease which was slightly more severe than that which resulted from infection with either agent alone. The two agents did not act synergistically to produce a markedly more severe diarrhea or death in our model. Significant interaction between the two agents was suggested by increased B44 *E. coli* counts in the ilea, increased villous atrophy in the ilea, and occasional incidence of ulcers in the
ceca of two dually infected calves. Factors which deserve further attention in our model are age at time of inoculation; dose of inocula; amount of feed; and timing of tests which detect effects of the agents used.


EFFECT OF ALTERING MICROBIAL AND ENVIRONMENTAL VARIABLES ON THE RESPONSE OF GNOTOBIOTIC CALVES TO DUAL INFECTIONS WITH ROTAVIRUS AND ESCHERICHIA COLI

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National Animal Disease Center, United States Department of Agriculture, Science and Education Administration, Agricultural Research Service, Ames, IA 50010\textsuperscript{1} and College of Veterinary Medicine, Iowa State, University, Ames, IA 50011\textsuperscript{2}

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The experiments reported here were attempts to produce fatal diarrhea in calves using dual infections with *Escherichia coli* and rotavirus under conditions in which monoinfections with either agent caused mild or no diarrheal disease. Several clinical, microbiologic and pathologic parameters were used to assess severity of disease and to gain insight into mechanisms of the interaction between the 2 infections. The basic model used has been described previously and did not produce fatal diarrhea. It consisted of gnotobiotic calves inoculated at 5 to 8 days of age with a strain of enterotoxigenic *E. coli* (ETEC) and a strain of rotavirus. The calves were observed for two days after inoculation. Variables were introduced into the basic model in attempts to identify factors contributing to fatal diarrhea. Variables which did not result in fatal diarrhea or which did not cause more severe disease in dually inoculated calves than in monoinoculated calves observed 2 days after inoculation were: 1) feed increased to 2 times baseline, 2) dose of ETEC increased to 10 times baseline, 3) calves inoculated when 2 days old, and 4) replacement of the ETEC strain with an *E. coli* that causes colisepticemia, and 5) use of a different strain of rotavirus. Increasing the observation period from 2 days to 6 days after inoculation resulted in occurrence of severe, watery, fatal diarrhea in 6 of 12 calves 32 to 72 hours after dual inoculation. The fatal diarrhea was associated with more intense colonization by the ETEC in the lower half of the small intestine. Histologic lesions were qualitatively similar between dually inoculated
and rotavirus monoinoculated calves, although there was significantly more severe atrophy of the ileal villi of dually inoculated calves. Those effects of the dual infections were consistent with synergistic interaction of the two infections. Inherent (presumably genetic) variation among calves in susceptibility to the two infections may account for the inconsistent demonstration of the synergistic interaction.
INTRODUCTION

A syndrome of severe, watery, often fatal diarrhea occurs among calves about 1 week old (1, 9, 10). Agents causally implicated in this syndrome include enterotoxigenic Escherichia coli (ETEC), rotavirus, coronavirus, Cryptosporidium sp., and combinations of these in mixed infections.

Experimental reproduction of watery, fatal diarrhea in gnotobiotic calves dually infected with E. coli and rotavirus has been reported (6, 8). However, calves were 1- to 2-days old and only one or two calves per experimental group were used. ETEC alone can cause watery, fatal diarrhea in 1- to 2-day-old calves but not in 1-week-old calves (6, 14). Attempts using dual ETEC and rotavirus inoculation to experimentally induce diarrhea in calves about 1 week old did not result in the severe, fatal syndrome (13, 15, 17). Furthermore, the severity of diarrhea was similar among dually infected and monoinfected calves (13, 15). However, enhanced colonization by ETEC in dual infections suggested some synergistic interaction between the two disease processes (13, 15).

We think that variables in addition to the infectious agents contribute to the watery, fatal diarrheal syndrome of calves about one week old (13). The purposes of this study were twofold: first, to test the hypothesis that synergistic interactions occur between infections with rotavirus and E. coli in dually infected calves; second, to study the effect of selected environmental and microbial variables on dual infections of gnotobiotic calves inoculated with both
E. coli and rotavirus. Six of 12 dually inoculated calves (5 to 7 days old, observed up to 6 days after inoculation) developed watery, fatal diarrhea. In contrast, none of the 12 monoinfected calves that age (observed for 6 days after inoculation) developed fatal or even clinically severe disease. Other variables tested did not consistently cause more severe disease in dual infections than in monoinfections.
MATERIALS AND METHODS

**Animals** The calves, both beef and dairy, were of varied genetic background. They were delivered by caesarian section and passed through a germicidal trap into an isolator (7). Calves were maintained in the isolator until death or necropsy. Nasal and rectal swabs were cultured for aerobic and anaerobic bacteria (7).

**Inocula** Two strains of *E. coli* and two strains of rotavirus were used (Table 1). The inocula prepared from the ETEC strain B44 (09:K30,99:NM) and the gnotobiotic calf-passaged rotavirus strain 76-23 [Nebraska Calf Diarrhea Virus (NCDV)] were from the same pools as were used in the basic model (13). *Escherichia coli* strain JL9 (078:NM) which causes colisepticemia in colostrum-deprived calves (5) was grown in Trypticase soy broth overnight at 37°C, and glycerol was added to a final concentration of 10% (volume/volume). The suspension was divided into 12 ml portions and frozen at -70°C until thawed for use. Two pools (prepared at different times) of rotavirus strain B641 (4) were prepared from aliquots of a single fecal sample of an experimentally infected gnotobiotic calf. Fecal aliquots were diluted 1:5 in 0.85% sterile saline, then twice were frozen at -70°C and thawed. They were centrifuged at 10,000 x g for 15 minutes. The supernatant fluid was decanted and centrifuged again at 10,000 x g for 15 minutes. That supernatant fluid was diluted 1:2 in 0.85% sterile saline, filtered through a series of Millipore (Millipore Corporation, Bedford, MA 01730) filters down to 0.45 μm pore size. The last filtrate was further diluted 1:2 in 0.85% sterile saline and passed through a .22 μm
Table 1. Source, characteristics, and concentration of inocula used to infect gnotobiotic calves

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Characteristics</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B44</td>
<td>Smith (14)</td>
<td>09:K30,99:NM, STa&lt;br&gt;STa&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 x 10&lt;sup&gt;9&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>JL9</td>
<td>Gay (5)</td>
<td>078:NM, Causes colisepticemia</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Rotavirus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCDV 76-23</td>
<td>Mebus (8)</td>
<td>---</td>
<td>1,410&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>B641a&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Gaul (4)</td>
<td>---</td>
<td>56,230&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>B641b&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Gaul (4)</td>
<td>---</td>
<td>27,020&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Produces heat stable enterotoxin (STa).
<sup>b</sup>Colony forming units/ml.
<sup>c</sup>50% cell culture infective dose/ml (12) [fluorescent focus assay (3)].
<sup>d</sup>Two different pools of B641 inoculum were prepared from aliquots of a single gnotobiotic calf fecal sample.
pore size filter. The last filtrate was divided into 10 ml portions and frozen at -70°C. Electron microscopy of the inocula showed characteristic rotavirus particles. A fluorescent focus assay of the rotavirus inocula (3) was used to determine the 50% cell culture infective dose (CCID$_{50}$) (12). However, the three rotavirus inocula were not adjusted to give the same challenge dose in terms of CCID$_{50}$.

**Clinical observations** The behavior, appetite, strength, fecal color, fecal consistency, rectal temperature, total plasma protein and packed red blood cell volume were determined and recorded for each calf at least once daily. Clinical determination of diarrhea was based on loose to watery fecal consistency at the time of observation rather than volume, frequency, or combinations of the three. Severity of clinical disease (Table 2) was based primarily on clinical judgement of diarrhea. Absence of significant signs was judged to be no disease; transient diarrhea was considered mild disease; persistent diarrhea accompanied by signs such as depression, weakness and inappetance was considered severe disease; and death of calves (with or without diarrhea) was fatal disease.

**Necropsy and histology** Calves that were alive at the end of the observation period were anesthetized with sodium pentabarbital in the isolator, removed and maintained in deep surgical anesthesia through the initial portion of the necropsy after which they were killed by exsanguination while anesthetized. The four sites sampled from the small intestine (13) were duodenum, about 20 cm from the pylorus; upper jejunum, about 100 cm from the duodenal site; ileum,
about 100 cm proximal to the ileocecal valve; and lower jejunum, one-half the distance from the upper jejunal site to the ileal site. Histologic samples were opened, stapled flat on index cards, and fixed in buffered 10% formalin. Fixed tissues were dehydrated, embedded in paraffin, cut 6 μm thick, and stained with hematoxylin and eosin. Fluorescent antibody techniques were used to detect K99 or rotavirus antigens in frozen sections (9). Small intestinal segments 10 cm long were collected aseptically for enumeration of bacteria by a spiral dilution method (Spiral Systems, Inc., Cincinnati, OH).

**Experimental design** The effect of 7 variables on the severity of diarrhea in calves infected with *E. coli* and/or rotavirus were studied (Table 2). For comparison the corresponding variables in the basic model (13) were to feed 360 ml evaporated milk mixed with 360 ml water twice daily, to inoculate at 5 to 8 days of age with 1 ml of the ETEC strain B44 and/or 10 ml of the rotavirus 76-23 inoculum, and to observe for 2 days after inoculation. All variables, except the one being selected in each experimental group, were as in the basic model.
Table 2. Experimental design and major clinical results in gnotobiotic calves with dual or monoinfections of *E. coli* and rotavirus

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of calves</th>
<th>Inocula</th>
<th>Clinical Disease&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rotavirus</td>
<td>E. coli</td>
</tr>
<tr>
<td>Basic model</td>
<td>5</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>76-23</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>---</td>
<td>B44</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>76-23</td>
<td>B44</td>
</tr>
<tr>
<td>Increased milk (2-fold)</td>
<td>1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>76-23</td>
<td>B44</td>
</tr>
<tr>
<td>Decreased age (2 days old)</td>
<td>2</td>
<td>76-23</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>---</td>
<td>B44</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>76-23</td>
<td>B44</td>
</tr>
<tr>
<td>Increased ETEC dose (10-fold)</td>
<td>2</td>
<td>---</td>
<td>B44</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>76-23</td>
<td>B44</td>
</tr>
<tr>
<td>Different <em>E. coli</em> (JL9)</td>
<td>2</td>
<td>---</td>
<td>JL9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>76-23</td>
<td>JL9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>B641b</td>
<td>---</td>
</tr>
<tr>
<td>------------------</td>
<td>---</td>
<td>--------</td>
<td>-----</td>
</tr>
<tr>
<td>Different rotavirus (B641b)</td>
<td>4</td>
<td>B641b</td>
<td>B44</td>
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<table>
<thead>
<tr>
<th></th>
<th>4</th>
<th>76-23</th>
<th>---</th>
<th>4</th>
<th>0</th>
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<th>0</th>
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<tr>
<td>Longer observation (6 days)</td>
<td>4</td>
<td></td>
<td>B44</td>
<td>4</td>
<td>0</td>
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<table>
<thead>
<tr>
<th></th>
<th>7</th>
<th>76-23</th>
<th>B44</th>
<th>4</th>
<th>1</th>
<th>0</th>
<th>2</th>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>4</th>
<th>B641a</th>
<th>---</th>
<th>4</th>
<th>0</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-day observation plus different rotavirus (B641a)</td>
<td>5</td>
<td>B641a</td>
<td>B44</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

- Mild = transient diarrhea; Severe = persistent, non-fatal diarrhea accompanied by other signs; Fatal = calf died (usually with diarrhea).

- The data on the basic model were previously reported (13). Calves were 1) fed 720 ml milk twice daily, 2) inoculated when 5 to 8 days old with rotavirus (10 ml) and E. coli (3 x 10^3 cells), and 3) observed for 2 days after inoculation. Except as noted, variables in other models were as in the basic model.

- Calves died without signs of diarrheal disease.
RESULTS

Clinical observations  Severe, watery, fatal diarrhea occurred among calves dually inoculated with rotavirus (76-23 or B641a) and ETEC strain B44 when the observation period was extended to 6 days (Table 2). The calves died (2/7 inoculated with E. coli B44 and rotavirus 76-23; 4/5 inoculated with B44 and rotavirus B641) 32 to 72 hours after dual inoculation. However, occurrence of fatal diarrheal disease in these groups was inconsistent as 6/12 dually inoculated calves remained healthy or had only mild, transient diarrhea and were necropsied 6 days after inoculation. Fecal excretion of rotavirus and E. coli was detected from both surviving and dying calves. Quantitative clinical data from calves in the two models with 6-day observation periods also indicated more severe disease among the dually inoculated calves (Tables 3 and 4). Six of nine fecal samples from fatal cases were less than 10% dry matter whereas only 2 of 107 samples from other calves in these models were similarly affected. Likewise, the packed red cell volume and total protein showed more consistent and more marked increase in samples from fatal cases than in other calves in these models.

In contrast, increasing the amount of milk fed, inoculating younger calves, increasing the dose of ETEC, using a different strain of E. coli, and using a different rotavirus inoculum (all with 2 day post-inoculation observation) failed to induce a striking difference between disease seen in dually inoculated and monoinoculated calves (Table 2). Only one calf (monoinoculated with ETEC B44 at 2 days of
Table 3. Incidence and average of fecal samples with < or = 10% fecal dry weight of monoinoculated and dually inoculated calves in the 6-day observation models

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Rotavirus</th>
<th>E. coli</th>
<th>None or Mild Disease</th>
<th>Fatal Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Incidence&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Average&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Calves</td>
<td>Samples</td>
</tr>
<tr>
<td>---</td>
<td>B44</td>
<td>0/4</td>
<td>0/24</td>
<td>23.8 (5.1)</td>
</tr>
<tr>
<td>76-23</td>
<td>B44</td>
<td>0/4</td>
<td>0/24</td>
<td>25.8 (5.5)</td>
</tr>
<tr>
<td>B641a</td>
<td>---</td>
<td>0/4</td>
<td>0/24</td>
<td>19.8 (3.6)</td>
</tr>
<tr>
<td>76-23</td>
<td>B44</td>
<td>1/5</td>
<td>2/29</td>
<td>8.1 (2.5)/23.0 (4.8)</td>
</tr>
<tr>
<td>B641a</td>
<td>B44</td>
<td>0/1</td>
<td>0/6</td>
<td>21.1 (6.6)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The ratios are of affected calves or samples to total calves or samples. An affected sample is one with percent fecal dry weight < or = 10. An affected calf is one which shed such a sample.

<sup>b</sup>The average is for affected samples/all other post-inoculation samples. Standard deviations are in parentheses.

<sup>c</sup>The difference between the affected average and the all other average is significant at P < or = 0.05, determined by Student's t test.
Table 4. Comparisons of percent packed red cell volume and total plasma protein (mg/dl) between monoinoculated and dually inoculated calves in the 6-day observation models

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>Inoculum</th>
<th>Rotavirus</th>
<th>E. coli</th>
<th>Clinical Disease&lt;sup&gt;a&lt;/sup&gt;</th>
<th>None or Mild</th>
<th>Fatal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Packed Cell Volume</td>
<td>76-23</td>
<td>B44</td>
<td>-1.8</td>
<td>(-10.7 to 8.6)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>B641a</td>
<td>---</td>
<td>4.4</td>
<td>(-6.1 to 12.5)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>76-23</td>
<td>B44</td>
<td>-4.0</td>
<td>(-21.9 to 22.9) (0.0 to 9.4)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>B641a</td>
<td>B44</td>
<td>1.7</td>
<td>(-2.8 to 0.0) (0.0 to 75.0)</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

---

| Total Protein | 76-23    | B44       | 2.4     | (-10.0 to 16.7) (6.8 to 8.5) | 7.6          | ---   |
|               | B641a    | B44       | -2.0    | 28.3                        | -3.3 to 0.0) (0.0 to 75.7) | ---   |

<sup>a</sup>Values are the average percent difference (range) between the last preinoculation sample and the post-inoculation samples. Positive values reflect an increase in hematocrit or total protein.
age) in those groups had severe, watery, fatal diarrheal disease. All others either died without signs of diarrheal disease; had mild, transient diarrhea; or remained healthy during the observation period. Quantitative clinical data also indicated that dual infections were not more severe than mono infections in these models. For example, incidence of FDW ≤ 10% after inoculation was 10/57 in monoinoculated calves and 1/50 in dually inoculated calves. No serum total protein from calves in these models were > or = 20% above the preinoculation value. A similar increase (> or = 20% above the preinoculation value) in packed red cell volume occurred in 3/63 samples from monoinoculated calves and 0/48 samples from dually inoculated calves.

Microbiology The small intestines of dually inoculated calves that died were more intensively colonized by ETEC than were intestines of surviving calves (Table 5). K99 antigen was more consistently found in frozen intestinal sections (Figure 1a), especially in lower jejunum and ileum, in the dually inoculated calves than in the monoinoculated calves (Table 5). Strain B44 was inconsistently isolated from venous blood and mesenteric lymph nodes of either monoinoculated or dually inoculated calves. B44 was isolated from the liver of only one calf (monoinoculated with B44 when 2 days old).

In contrast, *E. coli* strain JL9 proliferated in the small intestine of either monoinoculated or dually inoculated calves in numbers as high as 10^8 CFU/10 cm segment. It also was consistently
Table 5. Colony forming units (CFU) of E. coli and indirect fluorescent antibody (IFA) detection of K99 antigen in sections of small intestine of calves in the 6-day observation models

<table>
<thead>
<tr>
<th>Site</th>
<th>Inoculum</th>
<th>Clinical Disease</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rotavirus</td>
<td>E. coli</td>
<td>None or Mild</td>
<td>Fatal</td>
<td>CFU^</td>
<td>IFA^</td>
<td>CFU</td>
</tr>
<tr>
<td>Duodenum</td>
<td>---</td>
<td>B44</td>
<td>4.5 (2)</td>
<td>0/4</td>
<td>---</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>76-23</td>
<td>B44</td>
<td>3.8 (5)</td>
<td>0/5</td>
<td>---</td>
<td>0/1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B641a</td>
<td>B44</td>
<td>3.1 (1)</td>
<td>0/1</td>
<td>7.2 (2)</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Upper</td>
<td>---</td>
<td>B44</td>
<td>5.1 (2)</td>
<td>0/4</td>
<td>---</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
<td>76-23</td>
<td>B44</td>
<td>3.6 (5)</td>
<td>0/5</td>
<td>---</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B641a</td>
<td>B44</td>
<td>4.5 (1)</td>
<td>0/1</td>
<td>7.3 (2)</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Lower</td>
<td>---</td>
<td>B44</td>
<td>5.8 (2)</td>
<td>0/4</td>
<td>---</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
<td>76-23</td>
<td>B44</td>
<td>4.5 (5)</td>
<td>1/5</td>
<td>---</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B641a</td>
<td>B44</td>
<td>3.7 (1)</td>
<td>0/1</td>
<td>9.3 (2)</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>Ileum</td>
<td>---</td>
<td>B44</td>
<td>4.9 (4)</td>
<td>0/4</td>
<td>---</td>
<td>0/0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>76-23</td>
<td>B44</td>
<td>4.4 (5)</td>
<td>0/5</td>
<td>9.6 (1)</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B641a</td>
<td>B44</td>
<td>5.0 (1)</td>
<td>0/1</td>
<td>9.1 (2)</td>
<td>2/2</td>
<td></td>
</tr>
</tbody>
</table>

^CFU are expressed as log_{10} and are geometric means. The number of samples represented is in parentheses.

^bIFA (Indirect fluorescent antibody) results are the number of calves positive/number examined. Samples were taken only from calves which were necropsied as scheduled or shortly after death.
Table 6. Detection of rotavirus antigen by direct fluorescent antibody assay on frozen tissue sections of monoinoculated or dually inoculated calves

<table>
<thead>
<tr>
<th>Site</th>
<th>Observation Period</th>
<th>2 days</th>
<th>6 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Virus only</td>
<td>Dual</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td></td>
<td>4/5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8/9</td>
</tr>
<tr>
<td>Upper Jejunum</td>
<td></td>
<td>4/5</td>
<td>7/9</td>
</tr>
<tr>
<td>Lower Jejunum</td>
<td></td>
<td>4/5</td>
<td>6/9</td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td>3/5</td>
<td>3/9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of positive calves/number of calves examined. Samples were taken only from calves which were necropsied as scheduled or shortly after death. Rotavirus antigen was not detected from 1 calf in each of the first two columns, 2 calves in the third column, and 3 calves in the last column.
Fig. 1. Fluorescent antibody preparations of frozen sections of monoinoculated calf small intestine. a) K99\textsuperscript{+} \textit{E. coli} strain B44 can be seen in patchy layers closely associated with the villous epithelium. b) Rotavirus antigen in epithelial cells distributed in the upper part of the villi.
cultured from venous blood prior to necropsy and from liver and mesenteric lymph node taken during necropsy.

Rotavirus antigen was detected (Figure 1b) more consistently in sections from calves killed 2 days after inoculation than from calves killed 6 days after inoculation (Table 6). Rotavirus antigen tended to be detected more often in the upper half than in the lower half of the small intestine. Rotavirus particles were first detected in feces 32 hours to 4 days after monoinoculation with rotavirus and 32 hours to 5 days after dual inoculation. In both cases onset of shedding clustered around 1 to 2 days after inoculation. Shedding continued until necropsy. However, some monoinoculated and dually inoculated calves were necropsied before rotavirus particles were detected.

**Gross and histologic observations**  
Calves which died with severe diarrheal disease had dry, rough haircoats, loss of skin turgor, sunken eyeballs, and wet, matted perinea. The small intestine sometimes had fluid filled segments and was thin walled. The cecum and colon of these calves also was filled with fluid. The mildly or non-affected calves were not clinically dehydrated and had little material in the small intestine while the cecum and colon were filled with pasty, mucoid, and sometimes solid material.

Villous atrophy (short, blunt villi covered at least partly with cuboidal to squamous epithelium) was seen primarily in the duodenum, upper jejunum, and lower jejunum of rotavirus monoinoculated or dually inoculated calves (Figs. 2-4). At 2 days after inoculation, ileal villi of monoinoculated calves were shorter, but not significantly so,
Fig. 2. Villus height and crypt depth (mean ± s.e. for 10 crypts and 10 villi per calf, in μm). Previously published data (13) from the basic model are included for comparison.

* = significantly different from uninoculated (negative) control; † = significantly different from corresponding group at 2 days after inoculation; ‡ = significantly different from rotavirus monoinoculated group. Single symbol signifies the probability of a difference as large or larger by chance is \( \leq 0.05 \), double symbol signifies \( P \leq 0.01 \) (Student's t test)
Fig. 3. Jejunum, 2 days after inoculation.  a) Section from an uninoculated calf.  b) Section from rotavirus strain 76-23 monoinoculated calf.  Short, blunt villi are covered with cuboidal to columnar epithelium which is focally fused with epithelium on adjacent villi.  c) Section from a rotavirus strain B641 monoinoculated calf.  Comparable to b.  d) Section from an E. coli strain B44 monoinoculated calf.  Comparable to a)
Fig. 4. Jejunum, 2 days after inoculation. a) Dual inoculation with *E. coli* strain B44 and rotavirus strain 76-23. b) Dual inoculation with *E. coli* strain B44 and rotavirus strain B641. Short, blunt villi are covered with cuboidal to low columnar epithelium which is multifocally desquamated and fused.
than villi of control calves whereas ileal villi of dually inoculated calves were shorter than villi of monoinoculated calves and significantly shorter than villi of control calves (Figs. 2 and 5). At 6 days after inoculation, ileal villi had apparently recovered in length. Significant crypt hyperplasia was seen only in calves 6 days after inoculation (Fig. 2). Crypt hyperplasia was more consistent in the upper three sites than in the ileum.

There was mild to moderate, multifocal congestion of lamina proprial veins and capillaries in some sections of the small intestine of monoinoculated (either rotavirus or E. coli) calves. Congestion was frequently, but not always, associated with dilated lacteals and focal separation of epithelium from the lamina propria. Congestion was seen more frequently in sections from dually inoculated calves but was otherwise similar to that seen in monoinoculated calves. Differences in inoculum did not result in consistent, striking differences in cellular infiltrates in the lamina propria or exudates through the mucosa. Eosinophilic granulocytes were numerous in all groups; neutrophils were uncommon in monoinoculated calves, but tended to be more common in dually inoculated calves; while lymphocytic and histiocytic infiltrates were mild to moderate in all groups. Mild plasma cell infiltrates were seen only in calves 6 days after inoculation. Multifocal, petechial hemorrhages from villous tips and neutrophilic exudate from lateral and apical villous surfaces (Fig. 5) were seen inconsistently and only in dually infected calves. Patchy layers, microcolonies, and single adherent bacteria were inconsistently seen in the
four intestinal sites both from B44 monoinoculated calves and from dually inoculated calves. Continuous layers of adherent bacteria were inconsistently seen in the lower jejunum and ileum of dually inoculated calves (Fig. 5).

Epithelium covering aggregated lymphoid nodules (dome epithelium of Peyer's patches) was columnar in most sections, but there was mild, focal cuboidal metaplasia in some monoinfected and dually infected calves. Dome epithelium was rarely fused to epithelium of adjacent villi, but fusion occurred mainly in calves inoculated with rotavirus strain B641. Both eosinophilic granulocytes and neutrophils were seen in domes and in the dome epithelium of most calves, but the numbers of these cells were not always similar to the numbers seen in adjacent villi.

Postmortem change had occurred in calves inoculated with *E. coli* strain JL9. However, there was not notable villous atrophy. Veins of the lamina propria and submucosa were congested and contained bacteria.

**Microbiologic and serologic monitoring** Serum from all blood samples taken prior to necropsy were negative for neutralizing antibodies to bovine viral diarrhea (BVD) virus, infectious bovine rhinotracheitis virus and parainfluenza-3 virus. No BVD virus was isolated from any of the tissue samples. Thirty-three of 46 (72%) of the calves remained free of detectable aerobic and anaerobic bacterial contamination. Only calves given rotavirus inoculum shed virus and no calves shed detectable viral particles prior to inoculation.
Fig. 5. Ileum, 2 days after inoculation. a) Inoculation with rotavirus strain 76-23. Villi were not significantly shorter than were uninoculated controls and were covered with low columnar epithelium. b) Inoculation with *E. coli* strain B44 and rotavirus 76-23. Villi were significantly shortened and were covered with irregular, vacuolated columnar epithelium. Inset – Neutrophilic exudate near a small epithelial defect (arrow) and bacteria adherent in a continuous layer to the epithelial surface (arrowhead).
DISCUSSION

Following persistent attempts to replicate severe diarrheal disease, no variable studied resulted in consistent reproduction of watery, fatal diarrhea in dually inoculated calves but not monoinoculated calves. Increasing the observation period to 6 days resulted in inconsistent reproduction of the severe, fatal disease in dually inoculated calves but not monoinoculated calves which mimicked the syndrome in reported field cases (1, 9, 10). Variables which had no apparent effect or which did not permit differentiation between disease in dually inoculated and monoinoculated calves included increased feed, decreased age at time of inoculation, increased dose of B44 inoculum, use of a different strain of *E. coli*, and use of a different strain of rotavirus (all when calves were observed 2 days after inoculation). Several variables which were not tested may have contributed to the inconsistency seen in our models. First, important variables which were not well controlled in these experiments were the source and background of the calves. The calves were of varied genetic background, were both beef and dairy, and were obtained from both the Midwestern and Southern United States. It seems likely that calf-to-calf variation is a significant factor in the inconsistent response to the dual infections. In field cases of diarrheal disease, mortality is rarely 100% (1, 9, 18). Elimination or significant reduction of that variable would likely require many calves or possibly hyperovulation and embryo transfer to reduce the genetic variability of the gnotobiotic calves. An alternative would be to develop a model in
a different species such as mice which have multiple births, shorter gestation periods, and are better characterized genetically. Second, inocula which were prepared as lots then stored at -70°C were used throughout the course of these experiments. It is possible that the virulence, pathogenicity, infectivity, or combinations of those decreased with prolonged storage. This seems unlikely since the first deaths associated with the dual infection occurred over two years after the initial inocula were prepared, the B44 inocula contained a similar number of colony forming units throughout the experiments, and the rotavirus inocula alone continued to infect calves and cause typical lesions throughout the course of the experiments. However, onset of shedding of rotavirus tended to occur later in later calves. Third, different lots of evaporated milk were used during the course of these experiments. It is possible some contained sufficient antibody or other substance to modify the outcome of the infections. Use of a defined, synthetic diet (if available) or use of a single lot of diet would help control that variable.

Disease which resulted from mixed infections with *E. coli* and rotavirus previously has been described as significant or synergistic (6, 8, 15). Synergy is "the action of two or more substances, organs, or organisms to achieve an effect of which each is individually incapable" (11). Torres-Medina (16) recently described the interaction of dual infection with ETEC and rotavirus in one-day-old calves as additive. We also previously characterized the interaction of dual infections of ETEC and rotavirus in 5- to 8-day-old calves as additive
In contrast to our previous report, in the models with 6-day observation periods reported here, occurrence of fatal, watery diarrhea in dually inoculated calves was consistent with synergistic interaction of the dual infection. Clinical response (death being a striking endpoint) of dually inoculated calves with fatal diarrheal disease and intense colonization of the small intestine of those calves by the ETEC strain B44 are evidence of synergistic responses to the dual infection in those models. Similar effects did not occur or were uncommon among monoinoculated calves in the same models. Qualitative histologic differences between similar sections from monoinoculated or dually inoculated calves were slight, but included more severe villous atrophy and more extensive bacterial layers in the ileum, more frequent vascular congestion, and increased neutrophilic infiltration in the dually inoculated calves. Neutrophils seen in and over the domes of Peyer's patches in some sections of B44 infected calves (mono- and dually inoculated) are consistent with the report by Bellamy and Acres of neutrophilic caps over the domes in colostrum fed calves inoculated with ETEC (2). Subsequent to the completion of this work, the B641 inoculum was found to be contaminated with an astrovirus. Woode et al. (19) have speculated that viral infection of domes and dome epithelium may impair local immune response. The lesions of dome epithelium of some of the B641-inoculated calves in this report were similar to those described by Woode et al. (19). The higher incidence of death among calves dually inoculated with the B641 inoculum and B44 may be partially attributable to astrovirus infection with subsequent
impairment of immune function. However, despite the probable astrovirus contamination, severe, fatal diarrheal disease was inconsistently reproduced in calves dually inoculated with B641 and B44.

The distribution of rotavirus antigen in the small intestine of these calves is consistent with some previous reports (13, 15) but contrasts with others who suggested that rotavirus infections proceed in a rapid wave down the small intestine (8). First, rotavirus was detected more consistently from the upper small intestine than from the lower small intestine, regardless of the duration of infection. Second, rotavirus antigen was detected more consistently from calves killed 2 days after inoculation than from those killed 6 days after inoculation. And third, by six days, but not by 2 days after inoculation, there was evidence of crypt hyperplasia and regeneration of villous length at some sites. The calves used in our experiments were both older at the time of inoculation and were allowed to live longer after inoculation than were those of Mebus et al. (8). The infection may have been more persistent in the upper small intestine of our older calves. Alternatively, in our calves the lower small intestine may have been more resistant to infection than the upper small intestine.

Calves inoculated with the JL9 strain of E. coli had nondiarrheal disease that was characterized by rapid onset of high fever and death within 48 hours. It was consistent with the experimental coli-septicemia of colostrum-deprived neonatal calves (5). Mebus et al. (8) suggested that rotavirus infection permitted a nonpathogenic E. coli to
cross the mucosal barrier and cause septicemic disease. JL9 is a well characterized cause of colisepticemia and in our experiments was able to cross the mucosal barrier in the presence or absence of concomitant rotavirus infection. In contrast, ETEC strain B44 was inconsistently isolated from tissues other than intestine, even if the calf was moribund.

Because of inconsistent reproduction of the fatal disease, the mechanisms of the interaction between infections with rotavirus and ETEC were not significantly illuminated. However, some factors are apparent. Results reported here confirm other reports (13, 15) that rotavirus infection appears to enhance intestinal colonization by ETEC. The mechanism of the enhancement is uncertain, but may be due to changes in availability of cell receptors for the ETEC, changes in the microenvironment of the ETEC, or changes in intestinal motility and consequently in the ability of the host to eliminate the ETEC from the small intestine. As noted above, septicemia seems an unlikely explanation for severe, fatal disease. However, an effect of endotoxin in the fatal disease cannot be ruled out.

In conclusion this study provides strong evidence of synergistic interaction between concomitant rotavirus and ETEC infections of calves about 1 week old. However, reproduction of synergistic effects was inconsistent. It seems clear that factors, especially innate resistance of the calf (either genotypic or phenotypic), in addition to the infectious agents play an important role in the pathogenesis of the fatal disease.
LITERATURE CITED


GENERAL DISCUSSION AND SUMMARY

In this study we have presented evidence that the pathogenesis of ETEC infections may be influenced by the age of the host, characteristics of the ETEC strain and by concomitant infection of the host with rotavirus.

Age-dependent resistance to effects of ETEC have been observed (111, 148). The resistance may be partly attributable to the type of toxin secreted (111). The study reported here demonstrated that adherence mediated by the K99 pilus antigen may also be an age-dependent phenomenon in that K99* ETEC adhered less intensively in vitro to isolated intestinal epithelial cells of 6-week-old pigs than to cells of 2-day-old pigs. The phenomenon appeared to be pilus-specific since ETEC with the K88 pilus antigen adhered in equivalent, high numbers to cells from both 6-week-old and 2-day-old pigs. The specificity of the phenomenon suggests that receptors for the K99* ETEC are expressed differently in 6-week-old and 2-day-old pigs. Although this possibility remains untested, host-age dependent expression has been shown to occur with other intestinal cell surface products such as lactase and other disaccharidases (71).

While age-dependent resistance to adherence of isolated epithelial cells corresponds with development of resistance to ETEC infections of the host species, the inhibitory effect of capsule on adherence reported here contrasts with the effect of capsule in vivo. In the intact animal, the A-type mucoid capsule seems to enhance colonization and virulence of the ETEC (20, 21, 109, 151). In contrast, the capsule
confers no advantage (78) or is inhibitory to the ETEC's ability to adhere to isolated intestinal epithelial cells, as reported in these studies. The intensity of the inhibitory effect of capsule varied with the strain tested. The variability may be due to variable expression of capsular and pilus antigens. The mechanism of the *in vitro* phenomenon is uncertain, but may be due to interference with pilus-receptor interaction. This possibility should be investigated. Furthermore, the *in vitro* phenomenon suggests that the role of capsule in enhanced virulence and colonization of encapsulated ETEC *in vivo* may be due to a mechanism other than enhanced adherence to the intact epithelial cells.

Other possibilities suggested by Costerton et al. (24b, 24c) include stabilization of microcolonies, protection from soluble products in the lumen, protection from secreted products of the immune system, resistance to phagocytosis, and interaction with the mucous layer over the epithelial surface. Colonized intestinal epithelium washed vigorously with mucolytic agents remain colonized by ETEC (122) so it seems likely that an effect of mucus, *in vivo*, might be to trap, but not wash away the ETEC. Monosaccharides such as N-acetylgalactosamine and N-acetylneuraminic acid which are associated with the polysaccharide portion of mucus (120b) have also been identified as putative receptors for the K99 pilus antigen (95). The capsule may inhibit interaction between the pili and mucus during the initial stages of infection. After initial contact with the mucous layer, the ETEC may further penetrate, colonize the epithelium, and extend microcolonies under the relatively stable environment of the
host/parasite mucous gel. Such speculation provides a basis for investigating and comparing the inhibitory in vitro effect of capsule as described in this study with the in vivo effects which appear to promote colonization and virulence.

Finally, the occurrence of synergistic effects, exemplified in this study by death and increased colonization by ETEC in the ileum of dually infected calves confirms previous reports of significant interaction of E. coli and rotavirus infections (58, 102, 159, 170, 184). It extends these previous observations in that the severe, fatal disease was reproduced in several calves rather than one or two; it was reproduced with two different isolates of calf rotavirus; and third, it was reproduced in animals of varied genetic background. Of the reports cited above, only that by Wray et al. (184) used a similar number of animals. However, since severe diarrheal disease was reproduced inconsistently in both that report (184) and the studies reported here, mechanisms of the interaction were not significantly illuminated. Some suggestion that rotavirus infection enhanced colonization by the ETEC was apparent, and has also been reported to occur in conventional calves (159). However, since intense B44 colonization at an intestinal site rarely coincided with intense rotavirus infection (as detected by immunofluorescence), the effect of rotavirus on colonization seems likely to be indirect, regardless of the mechanism. The mechanism of enhanced colonization may be related to the ability of ETEC to attach to intestinal epithelium in the rotavirus infected calves. Adherence of ETEC in vitro to isolated epithelial cells from gnotobiotic calves
which were either uninoculated or inoculated with rotavirus failed to support that hypothesis (Runnels, work in progress). Rotavirus infections may alter motility of the intestine and enhance colonization by decreasing transit of material through the intestine. The crude methods used to assess transit of material through the intestine in this study neither supported nor refuted that hypothesis. Mucus is an important, but poorly defined component of normal intestinal mucosa. It stabilizes the microenvironment of the mucosal epithelium, provides a niche for intestinal microbes, and acts as a barrier to pathogenic microbes (24b, 50b, 120b). It is possible that the rotavirus infection altered production (either in quality or quantity) of the normal mucus gel, thereby permitting enhanced penetration of the mucus gel by B44 E. coli in some dually infected calves. Such a possibility was not examined in this study, but could be a fruitful area for future investigation.

Two general research areas arise from the disparate research topics in this study. First is the importance of receptor mediated phenomena in the pathogenesis of diarrhea caused by ETEC. Both studies of in vitro adherence suggest a need to further define the role of bacteria/epithelial cell interaction, especially at the receptor-ligand level. Further, work in progress suggests that site specificity seen in colonization of the intestine by K99+ ETEC may be due, in part, to differences in adherence of the ETEC to epithelial cells from the upper (low colonization and low in vitro adherence) and lower (intense colonization and intense in vitro adherence) intestinal sites.
Recently, both N-acetylneuraminic acid and N-acetylgalactosamine residues on complex carbohydrates have been identified as putative receptors for *E. coli* expressing K99 pili (95). Are these moieties also active in the intestine at either the bacteria/epithelial cell interface or the bacteria/mucus interface? Second is the potential for the genetic background of the host to modify its response to infections with ETEC. The variable adherence of ETEC to isolated epithelial cells, seen in the study on the effect of capsule and the variable response of gnotobiotic calves to mixed infections with ETEC and rotavirus were both suggestive of the importance of host genetics. It seems that the most useful experiments would be designed to resolve hypotheses arising from those areas. For example, are there identifiable differences in receptor populations or arrangement between neonatal and older pigs? Are there distinct swine populations that are either highly susceptible to K99 mediated adherence or resistant to such adherence? If such resistance exists, is it genetically mediated? Is the variable response of gnotobiotic animals to dual infections with ETEC and rotavirus genetically mediated? Is the variable response due to resistance (presumably genetic) to effects of rotavirus or to ETEC or to some unique combination of the two?

Finally, the mechanisms of interaction of rotavirus and ETEC remains an open question. An inexpensive, consistent model of the severe disease due to dual infection, but not single infections needs to be defined.
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APPENDIX A

METHODS FOR ISOLATING INTESTINAL EPITHELIAL CELLS FROM THE SMALL INTESTINE OF PIGS OR CALVES AND AN IN VITRO ADHESION ASSAY USING ISOLATED INTESTINAL EPITHELIAL CELLS AND ESCHERICHIA COLI

P. L. Runnels and S. M. Skartvedt
INTRODUCTION

The adherence of bacteria to biological substrates is an important part of the pathogenesis of many diseases. Several models which attempt to mimic this phenomenon in vitro have been described (1, 2, 4-8).

The cell isolation procedure described here has been adapted mainly from a technique described by Wilson and Hohmann (8), but contributions from other sources have been incorporated (4, 6, 7). It has been used to isolate intestinal epithelial cells from pigs 1 day to 6 weeks old and calves 1 day to 2 weeks old (5). The description is based on isolation of intestinal epithelial cells from a 2 day old pig.

MATERIALS AND METHODS

Buffers Prepare Krebs-Henseleit (KH) buffer (pH 7.4) in 10 liters of distilled water (6):

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.12 M</td>
<td>Sodium Chloride</td>
<td>70.1 g</td>
</tr>
<tr>
<td>0.014 M</td>
<td>Potassium Chloride</td>
<td>10.4 g</td>
</tr>
<tr>
<td>0.025 M</td>
<td>Sodium Bicarbonate</td>
<td>21.0 g</td>
</tr>
<tr>
<td>0.001 M</td>
<td>Potassium, Dihydrogen Phosphate ($\text{KH}_2\text{PO}_4$)</td>
<td>1.4 g</td>
</tr>
</tbody>
</table>

Adjust the pH with either concentrated sodium hydroxide or hydrochloric acid (usually the acid is needed), added drop-wise to the 10 L solution with stirring. Not more than 2 or 3 ml are usually needed. The buffer is stored at 4° C in bulk. A KH buffer with 1% D-mannose added (KHM) to inhibit type 1 pilus mediated adhesion of E. coli to epithelial cells can also be prepared.
Animals

The animals are held off feed, but allowed access to water, for at least 12 hours prior to euthanasia and removal of their intestines. They are killed with an overdose of phenobarbital (intravenous for larger animals and intracardiac for baby pigs). About 2 ml is usually sufficient for baby pigs, but 20 ml or more is required for calves. The animal is placed on its left side, so the right flank is exposed with the animal's head to the dissector's right. The skin is incised in a horizontal "U" shape, beginning at the ischium, extending cranially to the last rib; then ventrally along the rib, then caudally toward the inguinal region of the flank. The skin flap is reflected back, and the underlying musculature and peritoneum are similarly cut and reflected. The cecum is located, and the ileum is cut free at that point. The small intestine is separated from its mesentery, using blunt-tipped scissors, working from the ileum toward the duodenum. Care is taken to maintain the intestinal serosa intact, but small nicks in the gut do not adversely affect the isolation procedure. The intestine is cut again at the region of the ligament of Treitz, where the duodenum reflects around the root of the mesentery. There are 1 to 1 1/2 meters of intestine obtained from a 2-day-old pig. The isolated intestine is rinsed in a small volume of KH buffer to remove blood. It is placed in about 0.5 liter of KH buffer, and stored at 4°C for 1 hour. This allows autolytic processes to loosen the epithelial cells from the underlying basement membrane and each other.

Isolation apparatus

During the 1 hour wait, the apparatus may be set up (Figs. 1-3). A towel beneath the ring stands provides some
Fig. 1. Shaker and attachments: (Shaker assembly). The basic piece of equipment for the shaker assembly is the Vortex, Jr. Mixer. It can be turned on or off, and it is not pressure sensitive (1). The switch has been removed from this unit. The neoprene cup has also been removed. A bracket (2) was made to suspend the mixer from ring stands. A hole has been drilled and tapped at each corner of the base of the Vortex, Jr. Angle iron has been drilled to correspond to those holes, and bolted to the base. A steel rod has been welded to the angle irons. The Vortex, Jr. mixer has a rubber shock absorber (3), which has a nut and washer embedded in it. After several hours of use in the shaking procedure, the nut works its way loose and eventually falls out. The whole shock absorber with its clamp must be replaced as mentioned above in 8.f. An adapter (4) was made of 1/2" inside diameter iron pipe. It has a washer brazed to one end (not visible in the photo). The adapter is then bolted with the bolt (5) to the nut which is embedded in the rubber shock absorber in the Vortex, Jr. This bolt can work loose and disrupt harmonious shaking. A lock washer (6) helps keep it tight. The adapter has been drilled and tapped laterally near one end to receive a small "allen" bolt (7). A solid, threaded stud (8) has been drilled on the end which will receive the "gut holder". It has also been drilled and tapped laterally to receive a small "allen" bolt (7). A nut has been screwed onto the stud to act as a depth gauge when inserting it into the adapter. The "allen" bolt on either the stud or the adapter sometimes work loose during shaking, and must be retightened with the "allen" wrench (9).
Fig. 2. Shaking apparatus with the shaker assembly resting in the upper position. One holder (1) is ready to be inserted into the KH buffer (Note the rubber band fixing the upper edges of the gut sections in place). The other holder (2) is held by a spring clamp in a position which makes it easier to slide the gut over the steel rods. Three of the rods have been loaded with the partially inverted doubled thickness of gut as described in section 6. The Vortex, Jr. (3) is plugged into a Variac transformer (4), which in turn is plugged into another transformer (5), which is then plugged into the wall outlet (110 volts)
Fig. 3. Shaking apparatus with the shaker assembly in the lower "working" position. This gives a rough idea of the depth of KH buffer and coverage of the steel rods on the holder
Fig. 4. Holder for neonatal pig (1 day to 2 weeks old) intestine or neonatal (<1 week old) calf intestine. The holder is homemade, and is made of stainless steel parts. The steel rods (1) are 3/16" (about 0.4 cm) in diameter, and are 6 3/4" (about 17.1 cm) long. They are roughened at both the free and fixed end. The tip at the free end has been rounded and polished to remove rough edges. The steel disk (2) in which the rods are fixed is 5/16" (about .8 cm) thick, and 1 3/4" (about 4.4 cm) in diameter. The disk has eight holes spaced equidistant near the circumference. The steel rods have been inserted into the holes and welded in place. The disk has a hole drilled through its center to receive a 1/4" (about 0.6 cm) stud (3), which will fit into the drilled stud on the shaker assembly. The stud on the holder has been flattened on one edge to give a base for the "allen" bolt to fix against. The stud on the holder is threaded on the end which fits into the disk. It is fixed in place with one bolt above and one bolt below the disk (these bolts have not yet come loose during the shaking procedures)
Fig. 5. Alternative holder for intestine of 2 week old and younger pigs. The same apparatus as described for the stainless steel holder is used, but the soft copper helix [1/4 inch outside diameter (about 0.6 cm)] is inserted in place of the stainless steel holder. The helix has 4 coils, is 12.5 cm from top to bottom (excluding the vertical extension) and has a periodicity of about 3.2 cm
Fig. 6. Copper helix for shaking cells from calf intestine cells or cells from 6 week old pigs' intestines. The same basic apparatus is used, but scaled up in size. The threaded stud is removed from the shaker adapter and the helix made of 1/2" (about 1.3 cm) outside diameter soft copper tubing is inserted in its place. The calf gut would already have been loaded on that helix. The helix is about 11 cm, top to bottom (not including the vertical extension), with 4 coils in a periodicity of about 2.5 cm. The outside diameter of the helix is about 16 cm. When the helix was formed, the copper tubing flattened slightly. The flask containing KH buffer and the steel pan with ice and water are correspondingly larger.
noise reduction, and keeps the bench top cleaner. A stainless steel pan (20 cm wide x 35 cm long x 9 cm deep) is clamped to the ring stand. Two flasks, into which the cells are shaken, are placed in the pan. One is clamped to the ring stand so it is under the shaker; the other is left unclamped and out of the way. Crushed ice and about 1 liter of water are placed in the pan. The alignment of the shaker with the fixed flask is checked. (The shaker and homemade pieces are described in the figure legends.) The flasks are plastic 1000 ml graduated cylinders, which have been cut off at about the 500 ml mark. (Glass cylinders can be used, but they are difficult to cut and are prone to breakage.) The flasks are filled to about the 400 ml mark with KH buffer.

Isolation procedure The gut is removed from the KH buffer in which it was stored, and is placed into about 200 ml fresh KH buffer. An end is located and slipped over the steel rod about 2 cm, with the mucosal side in and serosal side out. A piece of silk suture (0) is tied around that end. As the gut is then gently pushed onto the rod, it inverts over the ligature and forms a double layer, with mucosal surfaces facing each other, and serosal surfaces facing the steel rod or the air. The gut is pushed on until the double layer is about 1/2 the way up the steel rod. It is then cut about 0.5 cm beyond the tip of the steel rod. The other rods on the holder are loaded in a similar manner, and the double layers are changed to single layers by sliding the gut the rest of the way onto the rod. The mucosal side is out, and the serosal side faces the steel rod. The loading process is much like
turning a sock inside out. When all the double layers have been unfolded, a rubber band is placed around them near the disk (Fig. 2).

The loaded holder is placed in the unattached flask and rinsed gently. It is then attached to the shaker while it is in the upper position (the shaker is supported freely in the upper position - it is not clamped). An "allen" bolt is tightened to fix the holder to the shaker assembly. The shaker is lowered to the lower position, and clamped in place, so the holder will not touch the sides or bottom of the flask.

The switch is turned on (the two Variac Transformers are hooked in series. The first is set at maximum output, and the second one (the one just before the shaker motor) is set at about 120, but adjusted up or down to get the system shaking harmoniously at high frequency, but low amplitude - about 3 mm). If the system gets out of the harmonious phase and the holder is oscillating in a wider arc, any of several things may be out of adjustment or broken. Try fixing it in the following order:

a. Turn down the transformer output until the shaker stops. Turn it up again gradually until the harmonious phase is reached.
b. The clamps on the ring stand may be holding the shaker assembly in a position which is less than optimum. Turn off the transformer, reposition the shaker, and turn on the transformer.
c. The flask may have shifted so that the steel rods or the steel disk are hitting the side or bottom of the flask. Reposition it.
d. The "alien" bolt may have loosened. Turn off the transformer, tighten the bolt, and turn the transformer back on.

e. The bolt (different than the "alien" bolt) which fixes the adapter assembly (Fig. 1) to the shaker may have loosened. Turn off the transformer, tighten the bolt, and turn on the transformer.

f. The springs, clamp, or rubber shock absorber in the Vortex Jr. shaker itself may be broken. These will have to be replaced before harmonious shaking can continue.

g. The motor in the Vortex Jr. may be wearing out. It will have to be replaced.

Shake the gut for 10 minutes. While one set is shaking, the other holder is loaded and placed in the unattached flask to wash. The gut which has been shaken for 10 minutes is discarded, and the holder reloaded. This process is repeated until the entire small intestine is used. [We formerly used 1/4 inch outside diameter (about 6 mm) soft copper tubing bent into a helix (Fig. 5) which would fit into the flasks. It was loaded in a manner similar to that described above, but since less total length was shaken at one time, we shook it for only 5 minutes.]

When the entire gut has been shaken, the cell suspension is transferred to centrifuge tubes and centrifuged at 200 X g for 10 minutes. The supernatant fluid is aspirated off the soft pellet. If several centrifuge tubes were used, the pellets were resuspended in minimal KHM buffer and pooled into 1 or 2 centrifuge tubes for washing 2
more times with KHM. The last supernate should be almost clear. The number of individual epithelial cells are counted microscopically in a hemacytometer to determine the concentration. An aliquot (or the whole amount, if desired) is adjusted to $2 \times 10^6$ cells/ml. They are stored at $4^\circ C$ and used on subsequent days without further washing. Trypan blue exclusion shows that about 50% of the isolated cells are viable after the procedure is complete. By the first day after isolation, none of the cells are viable by that test. Furthermore, by 1 day after isolation, many of the cells have disrupted, so that one has mixtures of brush borders and intact epithelial cells. This does not seem to affect the adherence assays. The total yield from a 2-day-old pig is about $10^8$ isolated cells.

**In vitro adhesion assay** The *E. coli* are grown on media which enhances production of the pilus in which we are interested. For K88+ *E. coli* the media is blood agar (5% sheep blood in blood agar base). For K99+ *E. coli*, the media is Minca IS (3) agar:

- $\text{KH}_2\text{PO}_4$: 1.36 g
- $\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O}$: 10.1 g
- Agar: 12.0 g
- Trace salts solution*: 1.0 ml
- Casamino acids: 1.0 g
- Distilled water: 1 liter

*Trace salts solution:

$\text{MgSO}_4\cdot7\text{H}_2\text{O}$: 10 g
Mn Cl₂·4H₂O 1.0 g
Fe Cl₃·6H₂O 0.135 g
Ca Cl₂·2H₂O 0.4 g
Distilled water 1 liter

Autoclave the Minca solution 20 minutes. Cool in a water bath to a temperature of 56 to 60° C. Add 10 ml sterile IsoVitaleX (1 vial), and then pour the plates. The cultures are streaked on the agar plates, which are then incubated overnight (18 to 20 hours) at 37° C.

The E. coli are suspended from the agar surface using about 1 ml of KHM buffer and glass "hockey stick" spreader or "rubber policeman". A 0.1 ml portion of the suspension is diluted 10 to 30 fold in KHM buffer. The approximate concentration of that dilute suspension is determined spectrophotometrically at 625 nm wavelength in a Bausch and Lomb Spectronic 20. (Standard curves of absorbance versus concentration had been prepared previously.) The volume of KHM needed to adjust the original suspension to about 2 x 10⁹ E. coli/ml can be calculated from the known concentration of the dilute suspension. The number of colony forming units in the working suspension is checked by plate counts, which are read the following day.

0.5 ml of the E. coli suspension (2 x 10⁹/ml) is mixed with 0.5 ml of the epithelial cell suspension (2 x 10⁶/ml). The mixture is agitated gently on a Vortex mixer for about 2 seconds. It is then incubated at 37° C on an oscillator (about 300 cycles/min.) for 1 hour. The mixture is then washed 2 times with KHM at about 200 X g in the centrifuge. The last pellet is suspended in about 0.2 ml KHM by flushing it gently in
and out of a Pasteur pipette. The suspension is then coded, so readers will not know its content. One drop of that coded suspension is placed on a glass slide, covered with a coverslip, and examined using a 100 X oil objective on a Leitz Orthoplan microscope that is equipped with Nomarski interference contrast optics. (Other means of visualization are possible, such as fixation and staining, or phase contrast optics.) All bacteria which are associated with an isolated brush border (Fig. 7a) or epithelial cell (Fig. 7b) are counted and recorded. Twenty such cells are counted by each of two persons (total of 40 cells per assay). The results are decoded and means ± standard deviation are calculated.

The adhesion assay is repeated on 3 days in a week, using the same epithelial cell suspensions, but fresh *E. coli* suspensions. New epithelial cell suspensions are prepared each week.
Fig. 7. (a) *E. coli* strain I26 (K99+) adherent to a brush border of a calf. There were an average of 62.7 adherent *E. coli* brush border in this preparation. (b) *E. coli* strain 263 adherent to an intestinal epithelial cell of a pig (average adherent bacteria were not determined for this preparation). These photomicrographs focus only on one plane of the preparation. To count adherent bacteria in the microscope, the plane of focus must be shifted up and down to include all adherent bacteria. (Unstained, Nomarski differential interference optics 1000 X)
LITERATURE CITED


APPENDIX B

PREPARATION AND USE OF $F_{ab}$ FRAGMENTS FROM IgG ISOLATED FROM RABBIT SERUM
INTRODUCTION

A molecule of immunoglobulin G (IgG) is composed of 4 peptide chains: two identical light (L) and two identical heavy (H) chains. Each L chain is bound to an H chain by a disulfide bond. The H chains are bound to each other by at least one disulfide bond. Thus, each IgG molecule is monomeric but has 2 antigen binding sites composed of the amino-terminal regions of the paired H and L chains. The enzyme papain cleaves a peptide bond in the H chains just on the amino side of the most amino-terminal disulfide bond between the H chains (5). There are three products of the enzymatic cleavage: Two identical F_{ab} (Antigen-Binding Fragments), each composed of one entire L chain bound via a disulfide bond to the amino-terminal half of an H chain, and an Fc (Crystallizable Fragment) composed of the carboxy-terminal half of each H chain still bound to each other via the disulfide bond.

Whole IgG molecules can bind two antigenic sites per IgG molecule. The antigenic sites can be on different antigen particles. The result of such inter-particulate binding between several antibody molecules and particulate antigens is agglutination (as with bacteria) or precipitation (as with soluble antigens). In contrast, an F_{ab} fragment has only one antigen binding site per molecule so solutions of F_{ab} fragments will not precipitate antigen or cause agglutination. Thus, F_{ab} can be used to block antigenic sites on an antigen when it is desirable for the antigen to remain in suspension or as single particles. Preventing adhesion of piliated E. coli to isolated intestinal epithelial cells by reacting the piliated E. coli with
anti-pilus $F_{ab}$ fragments (2) is an example of this use of $F_{ab}$ fragments which is relevant to the studies reported in this dissertation.
Isolation of IgG from whole serum  Specific anti-K88 or anti-K30 antisera [See manuscript 2, p. 37] were used as sources of IgG. IgG was isolated on a Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) liquid chromatography column according to the manufacturer's recommendations (1) as follows. The following buffer solutions were prepared in distilled water:

- 0.1 M Sodium Phosphate, pH 8.0
- 0.1 M Tris/HCl with 0.5 M NaCl, pH 8.5
- 0.1 M Sodium Acetate with 0.5 M NaCl, pH 4.5
- 0.1 M Glycine-HCl, pH 3.

Approximately 4.5 g of freeze-dried Protein-A Sepharose CL-4B was swollen for 15 minutes, then washed on a sintered glass filter with excess (about 1 L) 0.1 M sodium phosphate buffer, pH 8.0 (starting buffer). A slurry was prepared by suspending the swollen, washed gel in about 100 ml starting buffer. The slurry was poured into a 1 cm x 30 cm liquid chromatography column stepwise until all the gel was added. The column was sealed, allowing a small air bubble to remain. It was then inverted several times to resuspend the gel in the starting buffer. When the column was set upright, the gel packed by gravity into a volume of about 18 ml. The column was then washed sequentially with 10 column volumes of starting buffer, 10 column volumes of Tris buffer, 10 column volumes of acetate buffer and 10 column volumes of starting buffer. This procedure was followed to remove both unbound and bound contaminants. At the conclusion of the washing, whole serum
which had been dialyzed against 3 changes of 10 volumes of starting buffer (at least 2 hours/change) was carefully applied to the column. It was followed by 10 column volumes of starting buffer. Since the gel retained the IgG, the eluent from this stage of the procedure was discarded. The 0.1 M glycine buffer, pH 3.0 was applied slowly in the reverse direction as the serum had been applied. To ensure adequate binding and elution, the flow rate of application of serum and glycine buffer did not exceed 5 ml/hour. The eluent was passed through an ultraviolet absorption cell (Instrumentation Specialties Co., Lincoln, NE) and the fractions of the eluent which absorbed strongly at 280 nm wavelength were collected. There was a single, sharp absorption peak at that wavelength. Those fractions were pooled and immediately passed over a column of Sephadex G25 which had been equilibrated in 0.1 M sodium phosphate buffer, pH 7.0. This removed the glycine and suspended the isolated IgG in a neutral buffer. The final volume of IgG solution was about 3x the starting volume of serum. Therefore, the IgG solution was concentrated to about 10 ml over an XM50 ultrafilter (Amicon Corp., Lexington, MA) which excludes molecules larger than 50,000 daltons. The concentration of the IgG solution was determined using a radial immunodiffusion kit for IgG (Miles Laboratories, Inc., Elkhart, IN). The Protein A-Sepharose CL-4B column was washed and re-equilibrated as described above, while the Sephadex G25 column was washed and re-equilibrated with the 0.1 M phosphate buffer, pH 7.0.

Papain digestion of IgG A method described by Mage (4) which was a modification of Porter's method (6) was used to prepare the F_ab
fragments from the concentrated IgG. A special phosphate buffered saline (pH 7.3) was prepared: 0.01 M Sodium Phosphate, 0.15 M Sodium Chloride, 0.001 M EDTA, and 0.025 M Mercaptoethanol. The concentrated IgG was dialyzed in that buffer (3 changes, at least 2 hours per change). Following dialysis, 1 mg of mercuripapain (Sigma Chemical Co., St. Louis, MO) per 100 mg IgG was added to the solution. It was then incubated for 1 hour at 37°C. Proteolysis was stopped by adding iodoacetamide at a concentration of 0.030 M and a further 15 minutes incubation at 37°C. That solution was dialyzed in 0.1 M Sodium Phosphate buffer, pH 8 (starting buffer) as described above. After storage at 4°C overnight, Fc crystals had precipitated from the solution. The solution was centrifuged at 5,000 x g for 30 seconds in a microcentrifuge. The supernatant was collected and added to the equilibrated Protein A-Sepharose CL-4B column. The first protein rich fractions (containing F\textsubscript{ab} fragments) of the eluent were collected while the column was rinsed with starting buffer as described for isolation of IgG. Bound IgG and Fc fragments were eluted with the glycine buffer and the column was washed and re-equilibrated as described above. The fractions containing F\textsubscript{ab} were applied to a Sephadex G25 column as described above. The protein rich fraction (containing F\textsubscript{ab} fragments) was collected and concentrated 10-fold in a Minicon B15 concentrator (Amicon Corp., Danvers, MA).

The above procedure was repeated using both anti-K30 serum and anti-K88 serum. The F\textsubscript{ab} solutions were checked for purity by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
(SDS-PAGE), agar gel immunodiffusion (AGID), and immunoelectrophoresis (IEP). SDS-PAGE (reducing conditions) showed a single band at about 30,000 molecular weight. AGID showed very weak reaction with anti-Fab serum. IEP showed a single, smooth arc when the F_ab solution was reacted against anti-whole serum or anti-F_ab, but no arc against anti-Fc. When F_ab fractions of these sera were mixed with K30+E. coli, the anti-K30 F_ab caused very slight agglutination while the anti-K88 F_ab did not. The agglutination reaction with the anti-K30 F_ab was extinguished by dilution at about the same levels as was the ability of the F_ab to bind with the piliated E. coli. The cause of this agglutination was undetermined as impurity was not indicated by the assays noted above. Binding of F_ab to the piliated E. coli was detected by adding anti-F_ab serum to the F_ab/E. coli mixture. Agglutination by anti-F_ab serum was strong if the mixture was anti-K30 F_ab with K30+E. coli and was absent if the mixture was anti-K88 F_ab with K30+E. coli. The F_ab solutions were used in the experiments as described in the second manuscript (p. 37).

Subsequent to the completion of this project, a similar method for isolating F_ab fragments was described (2). The primary difference was in the method of enzymatic digestion: The papain was immobilized on a CH-Sepharose 4B column (2) rather than being free in solution. F_ab prepared by that method (2) showed a similar result (i.e., a single band on SDS-PAGE at around 30,000 molecular weight) as was seen by the method described above.
LITERATURE CITED


APPENDIX C

REVISION OF TABLE 2, MANUSCRIPT 3, WITH COMMENTS ON SIGNIFICANCE
OF THE RATIOS OF AFFECTED SAMPLES TO TOTAL SAMPLES
Table 2. Incidence, degree, and onset of fecal dry weights (FDW) < 15%

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Onset&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% FDW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>affected calves&lt;sup&gt;a&lt;/sup&gt;/ total calves</td>
<td>affected samples/ total samples</td>
<td>Onset&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control Rotavirus B44</td>
<td>3/5 (60)</td>
<td>10/29 (34)</td>
<td>29 (8-48)</td>
</tr>
<tr>
<td>Rotavirus</td>
<td>5/5 (100)</td>
<td>8/30 (27)</td>
<td>21 (8-32)</td>
</tr>
<tr>
<td>B44</td>
<td>3/4 (75)</td>
<td>8/21 (38)</td>
<td>13 (8-24)</td>
</tr>
<tr>
<td>Rotavirus + B44</td>
<td>4/4 (100)</td>
<td>17/24 (71)</td>
<td>18 (8-48)</td>
</tr>
<tr>
<td>All calves prior to inoculation</td>
<td>7/18 (39)</td>
<td>7/143 (5)</td>
<td>---</td>
</tr>
</tbody>
</table>

<sup>a</sup>Affected calves were those with fecal dry weights (FDW) < 15% for any sample taken after inoculation. Per cent values for the ratios are in parentheses.

<sup>b</sup>First fecal sample with FDW < 15%: Mean (range) hours after inoculation.
COMMENTS

The ratios of affected calves/total calves (AC/TC) and affected samples/total samples (AS/TS) were analyzed statistically by calculating the exact probability of differences occurring by chance between various ratios using the binomial distribution. The probability was at least 0.01 that the AC/TC ratio of the calves prior to inoculation differed from the post-inoculation AC/TC ratios of the rotavirus, B44, and rotavirus + B44 groups. However, the probability was about 0.09 (not significant) that the AC/TC ratios of the control group and the pre-inoculation group were different.

The AS/TS ratios of the control, rotavirus, and B44 groups did not differ from each other (P > 0.50). However, each was different (P > 0.01) from the AS/TS ratio of the rotavirus + B44 group and the pre-inoculation group. Since the interest was on interaction, the AS/TS ratios of the rotavirus and the B44 groups were added (sum = 8/30 + 8/21 = 408/630 = 0.65). That ratio does not differ from the AS/TS ratio of the rotavirus + B44 group. The conclusion to be drawn from this table is that although low fecal dry weight occurred most frequently among dually inoculated calves, it did so at additive rather than synergistic levels.
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