INFECTION OF LIGATED INTESTINAL LOOPS WITH HEMOLYTIC *ESCHERICHIA COLI* IN THE PIG*

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INTRODUCTION

THE PRESENCE of large numbers of hemolytic *E. coli* in the intestine of pigs with edema disease is now considered characteristic of the disease. Serological typing of these hemolytic *E. coli* has indicated that certain specific serotypes are usually involved. Some of the more commonly occurring ones are members of O groups 138, 139 and 141 (30). Likewise, weanling enteritis of pigs is associated with large numbers of hemolytic *E. coli* in the intestine. Serotypes having O antigens 138, 141 and 8 of hemolytic *E. coli* are commonly isolated from the intestine of pigs with this disease (30). Occasionally other strains of *E. coli* have also been incriminated. The serotypes of hemolytic *E. coli* found in edema disease and weanling enteritis are often present in the intestine of normal pigs, usually in smaller numbers (4).

In 1953, De and Chatterje (5) described a technique for studying the enteropathogenicity of *Vibrio cholerae* in rabbits. Test organisms were introduced into ligated loops of small intestine. Twenty-four hours later, pathogenicity was indicated in the isolated segment by distention with fluid and by necrosis and hemorrhage in the mucosa. This method was subsequently used to study *E. coli* isolated from diarrheal disease in man (6, 25, 31) and pig (14, 23, 26). Correlation between clinical and experimental enteropathogenicity was observed. Correlation of *E. coli* serotype with the positive loop reaction was equivocal. Most *E. coli* isolated from the small intestines of pigs with transmissible gastroenteritis of TGE cause a positive reaction in loops of rabbit intestine (26). Moon (23) has reported that many strains of *E. coli* isolated from the intestine of piglets with neonatal scours will cause fluid distention in inoculated loops of intestine of experimental piglets.

In this experiment, the enteropathogenicity of the serotypes 0138:K81(B):H14, 0139:K82(B):H1, 0141ab:K85(B):H4, and 0141acK87(B):HMN of hemolytic *E. coli* were studied in ligated loops of small intestine of weaned pigs. Immunological studies were also conducted.

MATERIALS AND METHODS

Animals

Pigs used in these experiments were purchased from farm droves, with the exception of four pigs which were caesarean-derived and raised in isolation. They were 1.5 to 3 months of age and of mixed breeding. Their feed consisted of a standard growing ration which was self-fed. Mice used in a toxicity study were a gray Bittner strain and weighed between 40 to 50 gm. at the time of use.

Bacteria

All the hemolytic *E. coli* strains used in these experiments were obtained from the Communicable Disease Center, Atlanta, Georgia. These serotypes were 0138:K81(B):H14, 0139:K82(B):H1, 0141ab:K85(B):H4 and 0141ac:K87(B):HNM. The organisms were maintained on tryptic soy agar[1] at room temperature in the dark. Those cultures used for intestinal loop inoculation were prepared by 18-hour incubation on 5% ovine blood agar plates at 37° C. Smooth, hemolytic, opaque colonies were selected and subcultured in tryptic soy broth; 1 to 1.5 ml. of 5- to 6-hour cultures of this broth were used to inoculate the ligated loops of intestine.

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Preparation of Ligated Loops

Pigs were fasted for 18 to 24 hours before surgery. Anesthesia was induced with sodium pentobarbitol given intravenously to effect or intraperitoneally, at a dose rate of 20 mg./kg. of body weight. Laparotomy was performed through the left flank.

The ileum was identified and used as a reference point to orient the preparation of loops. Six-inch segments of small intestine, in which no feed was present, were isolated by ligatures of silk, and inoculated with the desired agent via a 22-gauge needle. This procedure was repeated until five or six loops had been prepared at the desired level of the small intestine. The bowel separating inoculated segments served as absolute control loops; they were about six inches long. Peyer's patches were easily identified and were excluded or included in the loop as desired. The entire abdominal wall was closed by through-and-through vertical mattress sutures[2]. Twenty-four hours later, the pigs were anesthetized by the intraperitoneal administration of pentobarbitol sodium and killed by exsanguination. Necropsy was performed and loops were identified and examined. In order to rule out errors in inoculation, all infected loops were cultured on blood agar plates or tryptic soy agar slants and the live microorganisms isolated were tested with specific "OB" rabbit antiserums using the slide agglutination test (10). Thereby the presence and identity of the inoculated organism were presumably confirmed in each case.

Preparation of O and OK antigens

The O and OK antigens used for the immunization experiment were prepared using the methods described for the production of hyperimmune serum (9). They were not formalized.

Serology

E. coli agglutinin titers in pig sera were determined for both the O and OK antigen by the tube test described by Ewing (10). "OB" typing antisera used in the slide agglutination and tube test controls were obtained from the Communicable Disease Center, Atlanta, Georgia.

Protein Determination

The amount of protein in loop fluid was determined by the method of Lowry (21). Human serum was used as a standard[3].

Lactate Determination

Lactate analyses were done by the Barker and Summerson technique (2).

pH Determinations

The pH determinations on loop fluid were done with a pH Meter[4]. Loop fluid was collected in a beaker and determinations were made within two hours after the death of the pig.

Preparation of Loop Contents for Injection

Contents from 0138:K81(B):H14 infected loops to be used for injection into pigs and mice were centrifuged at 12,000 g. for 30 minutes. The supernatant fluid was decanted and frozen at −40° C. until used.

Histology

Tissues were taken from all loops for histological study. They were fixed in 10% formalin. Sections were cut at six microns and stained with hematoxylin and eosin.

RESULTS

Intestinal Ligated Loop Reaction to E. coli Infection

E. coli serotypes 0138:K81(B):H14, 0139:K82(B):H1, 0141ab:K85:H4 and 0141ac:K87(B):HNM were examined for enteropathogenicity in pigs by the ligated loop technique. Two serotypes were often tested in the same pig. The results of these trials are summarized in Table I.

The control loops consisted of those inoculated with sterile tryptic soy broth, and those uninoculated segments separating prepared loops. Their gross appearance

[2]Expanded Scale pH Meter, Model 76, Beckman Instruments, Inc., Fullerton, California.

[3]Lab-Trol, Dade Reagents Inc., Miami, Florida.

[4]Expanded Scale pH Meter, Model 76, Beckman Instruments, Inc., Fullerton, California.

TABLE I

SUMMARY OF ALL EXPERIMENTS IN WHICH LIGATED LOOPS OF INTESTINE WERE USED TO STUDY THE ENTEROPATHOGENICITY OF HEMOLYTIC *E. coli*

Agent	Number of pigs	Number of isolated loops prepared	Number of positive reactions
0138:K81(B):H14	32	130	125
0139:K82:(B)H1	4	12	0
0141ab:K85(B):H4	6	18	18
0141ac:K87(B):HNM	2	8	0
Sodium lactate (300 mg.)	3	5	0
Medium (Tryptic soy broth)	17	37	2*

*An organism giving a positive reaction in the slide agglutination test with *E. coli* 0138:K81(B) antisera was isolated from these loops.

at necropsy was unchanged from that observed at the time of preparation. This constituted a negative reaction (the only exceptions were two distended loops which were found to be infected with an *E. coli* giving a positive slide agglutination test with 0138:K81(B) antisera).

Serotypes 0138:K81(B):H14 and 0141-ab:K85(B):H4 regularly produced a grossly visible reaction when instilled into loops of small intestine (Figure 1). This reaction was confined to the infected loop and was characterized by fluid distention and usually by hyperemia. The fluid in the loop was turbid, very mucinous, often sanguineous and usually contained clots of precipitated mucus (Figure 2). Occasionally there was edema of the associated mesentery. All such reactions were considered "positive". The degree of distention of the positive loops with fluid varied but it was generally more pronounced in those prepared in the proximal part of the intestine. Peyer's patches were included in about one-half of the loops of each pig. Their presence did not appear to influence the response of the host (this included specifically immunized animals). The *E. coli* serotype used to inoculate each loop was recovered from all positive loops in pure or nearly pure culture.

Serotype 0139:K82(B):H1 and 0141-ac:K87(B):HNM produced no visible or distinct reaction in loops when compared to control or positive loops, even though bacteriological examination indicated infection. In some loops inoculated with these organisms, there may have been an increased amount of viscous mucus present. *E. coli* serotype 0139:K82(B):H1 has commonly been associated with edema disease.

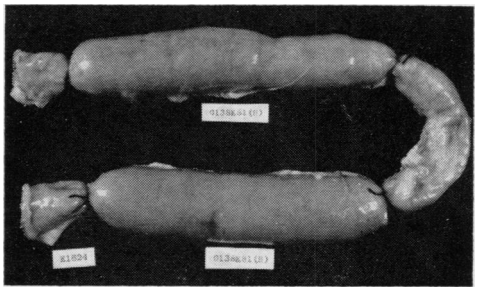

FIGURE 1. Ligated intestinal loops. Observe the fluid distention in two "positive" loops inoculated 24 hours previously with an enteropathogenic strain of 0138:K81(B) *E. coli*. Contrast this to the uninoculated control loop lying between them.

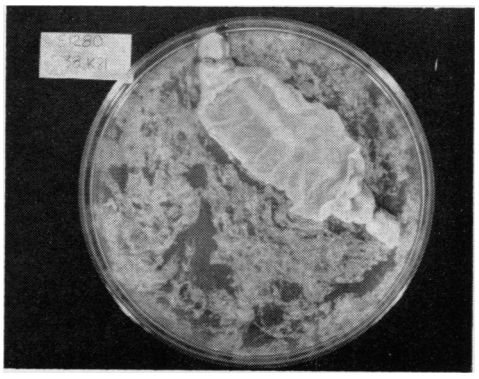

FIGURE 2. An opened "positive" loop inoculated 24 hours previously with an enteropathogenic strain of 0138:K81(B) *E. coli*. Note the shreds of mucus in the loop fluid.

Study of histologic sections of intestine from all loops revealed no remarkable

lesions or differences between positive and negative reactions. An exception occurred in three of four caesarean-derived pigs with loops infected with 0138:K81(B):H14. In these, there was edema and subintimal hyaline fibrinoid material in some of the small articles and veins of the entire small intestine. One month earlier these pigs had been infected orally with the same organism.

The protein content of positive loop fluid ranged from 0.5 to 1.2 gm./100 ml., with a mean of 0.7 gm./100 ml. These determinations were made on samples from 28 pigs. Therefore, the protein content of the loop fluid was no more than one-tenth the concentration of that of serum.

The pH measurements of the positive loop contents from 10 pigs infected with 0138:K81(B):H14 ranged from 7.8 to 8.4 and had a mean of 8.1.

Jenkin and Rowley (19) induced positive loops in rabbits by placing 20 mg. or more of sodium lactate in the ligated loop. In this experiment, the introduction of 300 mg. of racemic sodium lactate in 2 ml. doses into each of five loops had no effect. Lactate analysis of fluid from five positive loops infected with 0138:K81(B):H14 gave values of from 1 to 4mEq./1. These are in, or below, the normal range (1).

Other Necropsy Lesions

A mild serous of serofibrinous peritonitis was observed in some pigs. There were lesions in the esophageal cardia of the stomach of all pigs subjected to loop preparation, with the exception of four pigs in which Thiry fistulae had been prepared several weeks earlier. These lesions consisted of combinations of cornification, erosion and ulceration. There was edema of the submucosa in the immediately adjacent glandular cardia in 17 of 35 pigs with these cardial lesions. This varied in thickness from a few millimeters to 2 cm., and sometimes extended up to 5 cm. from the edge of the esophageal cardia.

Immunization Studies

An attempt was made to prevent the development of a positive reaction in 0138:K81(B):H14 inoculated loops by immunization with the OK and O antigen of this *E. coli* serotype. This procedure presented the possibility of demonstrating whether pathogenicity is associated with either the O or the K antigen. Fifteen pigs were used in this experiment. Five were immunized with OK antigen (live four-hour broth culture), five with O antigen (six-hour broth culture boiled for an hour), and five served as controls. Intravenous injections of 3, 5 and 10 ml. of appropriate antigen were given at 18, 11 and 4 days, respectively, before infected loops were prepared. The immunized pigs developed a high titer of serum agglutinins (Table II). The live organism (OK antigen) induced the highest serum O and K agglutinin titers. None of the pigs were protected against the development of a positive loop reaction.

Toxicity of Loop Fluid

Several investigators (15, 16, 28, 33) have been able to produce experimental edema disease by injecting pigs intravenously with the supernatant fluid of centrifuged intestinal contents taken from field cases of the disease. Gregory (17) reported an edema disease-like syndrome in mice inoculated with such material. The fluid from loops of eight pigs infected with 0138:K81(B):H14 was collected separately and prepared for injection by centrifugation; 10 ml. of supernatant from each sample was injected intravenously into each of eight pigs. No observable illness ensued. Negative results were also obtained when 50 mice were inoculated intraperitoneally with 0.25 to 1.0 ml. doses of this loop fluid.

Discussion

Clinical reports have clearly established that *E. coli* serotypes of 0138:K81(B) and 0141:K85(B) are associated with acute gastroenteritis in swine. The results of the loop tests correlate with these clinical findings. It appears that there is sufficient evidence to consider strains of these serogroups potential enteropathogens of swine. The mechanism for this pathogenicity remains to be determined.

The administration of these potentially enteropathogenic strains of 0138:K81(B) and 0141:K85(B), to weanling swine *per*

TABLE II

THE EFFECT OF IMMUNIZATION WITH THE O AND K ANTIGEN OF 0138:K81(B) ON THE INTESTINAL LOOP REACTION TO INFECTION WITH THIS ORGANISM

Pig number	Immunizing agent	Reciprocal serum titer*		Loop reaction
		K	O	
1257		128	4096	Positive (5/5)**
1258	Live	64	2048	Positive (5/5)
1259	0138:K81(B):H14	128	8192	Positive (5/5)
1260	(OK antigen)	256	8192	Positive (4/5)
1261		128	8192	Positive (2/5)
1262		64	1024	Positive (5/5)
1263	Boiled	32	2048	Positive (5/5)
1264	0138:K81(B):H14	64	2048	Positive (5/5)
1265	(O antigen)	32	2048	Positive (5/5)
1266		64	4096	Positive (5/5)
1267		0***	0	Positive (5/5)
1268		0	0	Positive (4/4)
1269	Control	0	0	Positive (4/4)
1270		0	0	Positive (4/4)
1271		0	0	Positive (4/4)

*At time of loop preparation.
**Enclosed in parentheses are the ratios of the number of positive loops to the number prepared.
***O indicates no titer at 1:8 dilution.

os usually results in no discernible disease (16, 18, 24, 29). Primary oral infection of caesarean-derived swine with potentially enteropathogenic strains has sometimes resulted in a mild clinical illness in a few pigs, manifested by slight depression and a slight diarrhea (27). In contrast, the ligated loop procedure seems to be a reliable method of regularly producing a form of experimental infection in which a well-defined host reaction is observable. Apparently, the confinement of an enteropathogenic *E. coli* in a ligated segment of bowel allows it to proliferate at one site and express its disease producing potential. The importance of intestinal motility in controlling the bacterial flora is thereby illustrated.

The pathogenesis of the reaction in the positive loop in the pig is not clear. It seems that multiplication of the *E. coli* is necessary for the positive reaction (19). The gross evidence for an inflammatory response in the host are the hyperemia, although this is not a constant feature, and possibly the transit of fluid into the lumen. With the exception mentioned earlier, the histologic examination of positive loops 24 hours after preparation and infection did not reveal any remarkable inflammatory changes, either cellular or vascular. Smith (29) has pointed out the absence of inflammatory changes in the intestines of piglets with diarrhea supposedly caused by *E. coli*. In weanlings, however, he did observe enteritis associated with *E. coli* infection. Possibly, if the loops were examined at earlier time intervals, inflammatory changes might be detectable. Moon (23) described inflammatory changes in the mucosa of positive loops in piglets three hours after inoculation with the enteropathogenic *E. coli*. In three-week-old pigs, Gyles (14) has observed eosinophilic fluid in the lamina propria and submucosa of positive loops infected with *E. coli*.

In rabbits, *Vibrio cholerae* infection induces positive loops within a few hours (12). At this time, the exit of fluid into the lumen of the infected loop is associated with an acute inflammatory reaction in the mucosa, characterized by hyperemia, edema of the lamina propria, and neutrophil infiltration. Keusch (20) has recently described increased vascular permeability in the venules of the lamin propria in experimental cholera in rabbits. The intact epithelial lining bars the passage of protein into the intestinal lumen

and the loop fluid contains only about 0.5 gm./100 ml. This agrees roughly with the value of 0.7 gm./100 ml. observed in the *E. coli* infected pigs. The low protein content of the loop fluid indicates that some care should be taken in deciding if there is fibrin in an intestinal "exudate". Necrosis of the epithelium would seem to be a prerequisite for fibrin exudation since the intact epithelium and its basement membrane apparently prevent the passage of large protein molecules into the lumen.

Jenkin and Rowley (19) suggested that lactic acid, bacterial mucinase and endotoxin were involved in the pathogenesis of positive loops in *Vibrio cholerae* infected rabbits. High levels of lactic acid in positive loops was considered especially significant. This view was challenged by Formal *et al.* (12), who reported that lactic acid did not rise above normal values until after distention was present and necrosis of the epithelium had occurred. Both of these workers, however, were able to induce positive loops, albeit inconsistently, in rabbits by placing 20 mg. or more of sodium lactate in the isolated lumen. These results could not be duplicated in pigs even though the amount used in this experiment, 300 mg., was well in excess of that required to produce a positive loop in a rabbit.

The transit of fluid into a positive loop could conceivably result from increased osmotic pressure within the lumen of the isolated segment generated by the growth of the enteropathogen. Breakdown of macromolecules in the lumen and an increase in bacteria and their products at a rate exceeding molecular absorption would raise intraluminal osmotic pressure and attract fluid. Observations, however, have indicated that no such osmotic gradient is present in positive loops (21, 23).

It has been suggested that a disturbance of the mechanism responsible for sodium absorption in the intestinal epithelium would account for the pathogenesis of the rice water stool of human cholera, and hence presumably the positive loop reaction also (11, 13). The question, however, has not been satisfactorily answered. Leitch (21), in a study of *V. cholera* toxin-induced positive loops in rabbits, observed concentrations of sodium in loop fluid to be greater than in plasma. Nevertheless, the electrical potential across the intestinal wall of the positive loop, presumably the result of active sodium transport, was maintained. He found the most important disturbance of ion movements to be secretion of excessive amounts of bicarbonate into the lumen.

The intravenous immunization of swine with either a boiled or a live culture of enteropathogenic *E. coli* did not prevent the positive reaction in the isolated intestinal loop. Taylor *et al.* (31) reported equivocal results in similar experiments with human enteropathogenic *E. coli* in rabbits. One explanation for this failure to protect is that antibody does not reach the lumen of the bowel where infection occurs. Jenkin and Rowley (19) were able by immunization to prevent the positive reaction in loops infected with *V. cholerae* in rabbits. It may be that the injurious action of *V. cholerae* is different than that of *E. coli*. Bacteria-free filtrates of this organism will induce positive loops in rabbits (7, 8). It seems likely that classical immunization techniques will be of little value in preventing enteritis or at least diarrhea caused by *E. coli*.

Success has been reported in reproducing edema disease by injecting intravenously the extracts of intestinal content from pigs with field cases of edema disease (15, 16, 28, 33). In the experiments reported here, no evidence for a toxic agent could be found in the 0138:K81(B):H14 infected loop contents. If this serotype is capable of causing edema disease, factors other than growth and multiplication of this hemolytic *E. coli* within the intestinal tract are necessary for the production of an edema disease "toxin".

Summary

Hemolytic *E. coli* serotypes 0138:K81(B):H14, 0139:K82(B):H1, 0141ab:K85(B):H4 and 0141ac:K87(B):HNM were examined individually for enteropathogenicity in pigs by inoculation of intestinal loops isolated by ligatures. Twenty-four hours later, a positive reaction in the infected loops was characterized by fluid distention and usually hyperemia. Serotypes 0138:K81(B):H14

and 0141ab:K85(B):H4 were found to be consistently enteropathogenic in this test, whereas serotypes 0139:K82(B):H1 and 0141ac:K87(B):HNM were not.

Fluid from positive loops infected with 0138:K81(B):H14 was non-toxic to other pigs and mice when administered parenterally.

Immunization of pigs with live or heat-killed 0138:K81(B):H14 did not prevent the development of a positive reaction in ligated intestinal loops infected with the same organism.

Résumé

Dans le but d'en déterminer la pathogénicité pour l'intestin du porc, on étudia séparément des souches hémolitiques d'*E. coli* des groupes sérologiques 0138: K81 (B): H 14, 0139: K82 (B): H 1, 0141 ab: K 85 (B): H 4 et 0141 ac: K 87 (B): HNM. On inocula chaque souche dans une anse intestinale ligaturée. Vingt-quatre heures après l'inoculation, on observait une réaction positive: l'anse infectée était distendue par un liquide trouble et apparaissait habituellement congestionnée. Au cours de cette même expérience, les souches appartenant aux types sérologiques 0138: K 81 (B): H 14 et 0141 ab: K 85 (B): H 4 s'avérèrent toujours pathogènes, alors que celles appartenant aux types 0139: K 82 (B): H 1 et 0141 ac: K87 (B): HNM se comportèrent différemment.

Le liquide provenant des anses intestinales et réagissant positivement à la souche 0138: K 81 (B): 14 ne se montra pas toxique pour les autres porcs et pour les souris auxquels on l'inocula. L'immunisation des porcs avec une souche 0138: K 81 (B): H 14, vivante ou tuée par la chaleur, n'empêcha pas une réaction positive au niveau des anses intestinales infectées avec une souche identique.

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A New Influenza Virus Infection in Turkeys.
I. Isolation and Characterization of Virus 6213

1. Table III: Thermo-inactivation of Virus 6213
   Virus Exposed to 4°C had a $CEID_{50}$ at Day 44 of log 6.4, and at Day 75 of log 4.2.
2. Legend to Table IV: Serological Identification of Virus 6213 by Hemagglutination-Inhibition Tests.

Infl. AH0/1/2—Influenza type A humanus, serotype 0, 1 or 2
Infl. AS—Influenza type A suis
Infl. AE1/2—Influenza type A equi, serotype 1 or 2
Infl. AA1 to AA6—Influenza type A avium, serotype 1 to 6
Infl. BH—Influenza type B humanus
Serum titers expressed in reciprocals of the serum dilution.
*neg.—No inhibition at dilutions 1/10 or higher.
N.T.—Not Tested