Elicitation of Enteroluminal Neutrophils by Enterotoxigenic and Nonenterotoxicogenic Strains of *Escherichia coli* in Swine

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In intact neonatal piglets, two strains of enterotoxigenic *Escherichia coli* (ETEC), which could adhere to epithelial cells and thus colonize the small intestine, attracted greater numbers of neutrophils into the lumen and wall of the intestine than did a nonenteropathogenic strain of *E. coli*. Ligated loops of small intestine in 8-week-old pigs were used in attempts to identify the attributes of ETEC involved in stimulating an increased enteroluminal migration of neutrophils. A nonenteropathogenic strain of *E. coli* did not attract neutrophils into the intestinal lumen in this model. However, three of the five ETEC strains tested did so. The three positive strains all produced heat-stable enterotoxin type b (STb). Neither of the negative ETEC strains produced STb. An STb-containing culture supernatant prepared from a strain of *E. coli* which contained an STb plasmid did not attract significantly more neutrophils than did a control supernatant prepared from the same strain of *E. coli* without the plasmid. The ETEC strains which attracted neutrophils in loops did not associate intimately with loop villi more consistently, nor did they grow to higher numbers in loops than strains which did not. It was concluded that there are increased numbers of neutrophils in the intestinal lumen during ETEC infection of newborn pigs. However, attempts to identify the attribute(s) of ETEC responsible for eliciting enteroluminal neutrophils were not successful.

Neutrophils are associated with many types of intestinal infections. Tissue damage generated by invasive bacteria, viruses, or parasites attracts neutrophils (12). Increased numbers of neutrophils in the lumen of the gut in calves suffering from enterotoxigenic *Escherichia coli* (ETEC) infection have also been described (2). The histopathological lesions in these calves were relatively mild (villous blunting, focal degeneration of enterocytes, and exfoliation of epithelial cells), yet emigration of neutrophils into the lumen was observed. A noninvasive intestinal disease such as that caused by ETEC would presumably attract neutrophils into the lumen of the gut by methods other than gross tissue injury. The chemotactic agents which are effective in such a noninvasive disease are unknown. Beneficial effects of the enteroluminal neutrophils to the host have been proposed (1–3, 11, 20), although others feel that neutrophils in the intestine are deleterious (9).

Bellamy and co-workers (1–3) and Sellwood (20) suggested that neutrophils play a protective role in the intestinal lumen. Bellamy and Nielsen developed the concept of immune-mediated emigration of neutrophils into the lumen of the gut (3). According to Bellamy, the presence of an antigen in the lumen of the gut would attract neutrophils, provided that the host possessed circulating specific antibody. He also showed that the presence of enteroluminal neutrophils was associated with a luminal bacterial population which did not proliferate; he believed that phagocytosis on the mucosal surface was an important protective mechanism in the small intestine (1). We tried to duplicate the immune-mediated chemotaxis experiments of Bellamy but were unable to do so for reasons that are as yet unclear. The concepts and results presented by Bellamy and co-workers merit additional study.

Sellwood collected immune and nonimmune colostrum, monitored the in vivo protection such colostrum gave to piglets (20), and concluded that immune colostrum could function in vitro both by preventing adhesion of the bacteria to gut epithelial cells and by promoting phagocytosis. He also proposed that these mechanisms were active in vivo, provided that emigration of neutrophils across intestinal epithelium occurs in newborn piglets.

The objectives of the work reported here were (i) to determine whether the presence of *E. coli* cells causes neutrophils to emigrate into the intestinal lumen of intact newborn piglets; (ii) to determine whether *E. coli* strains vary in their ability to attract neutrophils into the lumen of ligated intestinal loops in swine; and (iii) to define some of the attributes of *E. coli* that are involved in attracting neutrophils into the intestinal lumen.

We report here differences between ETEC and non-ETEC strains in the attraction of enteroluminal neutrophils in neonatal piglets. In ligated loops, these strain differences are not attributed to any chemotactant activity of enterotoxin.

**MATERIALS AND METHODS**

Four different experiments were performed (Table 1). Reference is made to these in the text below when appropriate.

**E. coli strains and preparations.** Pertinent characteristics of the strains of *E. coli* used are listed (Table 2). Each strain of *E. coli* was grown by inoculating 10 ml of Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) or brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) and shaking for 18 h aerobically at 37°C. The only strains inoculated into BHI were those cultured specifically for heat-stable enterotoxin b (STb) production (see below). Supernatants required were prepared by centrifuging the bacterial growths at 15,300 × g for 30 min at room temperature, followed by filtering them through a membrane filter (pore size, 0.22 µm; Millipore Corp.).

Five separate cultures of *E. coli* 263 were killed by five different methods: application of moist heat, β-propiolactone, mercurochrome, phenol, or Formalin. The culture killed by moist heat was exposed to steam for 65 min. Another culture of strain 263 was made 0.2% β-propiolactone and allowed to sit for 10 min before being washed with TSB. Killing with

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Inocula were injected with a 26-gauge needle. Each inoculum contained 6 ml of test material plus 3 ml of 320 mosM mannitol. The test material was either an overnight TSB culture of *E. coli* (first six strains in Table 2) or a supernatant from a BHI overnight culture of the *E. coli* (last two strains in Table 2; see STb preparation below). The mannitol ensured that fluid would remain in the intestinal segment.

After the last ligated loop had been inoculated, the small intestine was returned to the abdominal cavity. The abdominal wall was closed, and the animal was allowed to recover from surgery. The pigs were killed at either 2, 4, or 6 h after surgery by injection of sodium pentobarbital IV. Sample collection is described below. After the samples were collected, the rest of the pig carcass was inspected for the presence of any complicating factors, such as purulent pneumonia, which might competitively attract neutrophils. No such factors were found.

Fixing, staining, and identifying neutrophils in smears of intestinal fluids (experiments 1, 2, and 4). The smears were allowed to air dry and were then fixed for 1 min in a solution of 1 part 10% buffered neutral Formalin–3 parts 96% ethanol. The fixed smears of intestinal fluid were stained for peroxidase-positive cells (25). Fixed smears were rinsed in distilled water and counterstained with 1% toluidine blue.

Microscopically, neutrophils appeared as lobulated blue nuclei associated with fine brown granules. Eosinophils were distinguished by their very large brown peroxidase-positive granules. Many of the cells observed were larger than neutrophils, had round nuclei, lacked granules, and were assumed to be mostly epithelial cells. Two hundred cells were examined from each smear, and the percentage of cells that were neutrophils was recorded.

**Total neutrophils per loop** (experiment 2). Mucolexx (Lerner Laboratories, New Haven, Conn.) (1 ml) was added to each 4.5-ml sample of intestinal fluid to facilitate the use of a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.). The volume of fluid present in each ligated loop was recorded. The number of cells per milliliter was converted to cells per loop, taking into account the addition of Mucolexx. Multiplication of the number of cells per loop by the percentage of neutrophils in that loop yielded a value for the total number of neutrophils per loop.

**Processing of solid tissues** (experiments 1 to 4). Tissues were placed in Bouin-Holland fixative immediately after necropsy. This fixative is similar to Bouin fixative (21), except that it is also made 2.5% cupric acetate. The tissues remained in this fixative for 18 h. They were washed three times in 70% ethanol and then three times in 50% ethanol. These tissues were dehydrated, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin. All histological sections (two per loop) were encoded, evaluated microscopically by the following criteria, and decoded, and the results were summarized. The criteria for grading the

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Experimental animals (status of intestine)</th>
<th>n</th>
<th>Question</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Neonatal piglets (intact intestine)</td>
<td>15</td>
<td>Do ETEC and non-ETEC strains attract neutrophils into the intestinal lumina of neonatal piglets?</td>
</tr>
<tr>
<td>2</td>
<td>8-week-old pigs (ligated loops)</td>
<td>6</td>
<td>Will all strains attract neutrophils in a system in which they all attain high numbers? Will killed <em>E. coli</em> cells attract neutrophils?</td>
</tr>
<tr>
<td>3</td>
<td>8-week-old pigs (ligated loops)</td>
<td>8</td>
<td>Do positive and negative strains attain similar numbers in loops?</td>
</tr>
<tr>
<td>4</td>
<td>8-week-old pigs (ligated loops)</td>
<td>6</td>
<td>Is STb an attractant?</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype</th>
<th>Enterotoxin</th>
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<tbody>
<tr>
<td>123</td>
<td>O43:K:--H28</td>
<td>None</td>
</tr>
<tr>
<td>263</td>
<td>O8:K87, K88:19</td>
<td>LT, STb</td>
</tr>
<tr>
<td>431</td>
<td>C101:K30(A), K99:NM</td>
<td>STa</td>
</tr>
<tr>
<td>987</td>
<td>O9:K103, 987P:NM</td>
<td>STa, STb</td>
</tr>
<tr>
<td>A-1 (1288)</td>
<td>O149:K91, K88:H10</td>
<td>LT, STa, STb</td>
</tr>
<tr>
<td>Troyer (1459)</td>
<td>O9:K35, K99:NM</td>
<td>STa</td>
</tr>
<tr>
<td>HB101</td>
<td>K-12 (rough)</td>
<td>None</td>
</tr>
<tr>
<td>HB101(pRAS-1)</td>
<td>K-12 (rough)</td>
<td>STb</td>
</tr>
</tbody>
</table>

mercurichrome was done by first centrifuging a culture of strain 263 as described above. The supernatant was decanted, and 6 ml of mercurichrome was added to the pellet and allowed to react for 1 h. The solution was then centrifuged again, and the pellet was washed three times in sterile TSB. The final pellet was resuspended in 6 ml of TSB. Killing with 3% phenol or 10% Formalin was done by the mercurichrome procedure. The effectiveness of these treatments in killing the bacteria was verified by plating.

**Intact neonatal piglets** (experiment 1). Fifteen neonatal piglets delivered by cesarean section and deprived of colos- trum were used. Each piglet was given 20 ml of pooled normal pig serum intraperitoneally (24). Nine of the piglets each received an oral inoculum of 8 × 10⁹ cells of *E. coli* 123, 431, or 987 at 6 to 8 h of age. The remaining six control piglets received only 5 ml of TSB. One pig per *E. coli* strain and two control piglets were necropsied at 2, 8, and 16 h postinoculation.

At necropsy, two locations were chosen for fluid sampling. One location was in the ileum, 5 cm proximal to the ileocecral ligament. The other location was at the tip of the spiral colon. (This colon fluid was sampled to find neutrophils washed down from the small intestine.) A piece of intestine approximately 5 cm long was ligated off at each location. Saline (2 ml) was injected into each of these loops and then withdrawn. Smears of this saline flush were fixed and stained for peroxidase-positive cells as described below. The smears were then examined microscopically to determine the percentage of neutrophils present in the cells of this flush.

Tissue samples were taken at the upper jejunum (5 cm distal to the ligament of Treitz), the ileum, and the mid-small intestine (lower jejunum). These were processed and examined microscopically for neutrophils and layers of adherent bacteria.

**Intestinal loop procedures** (experiments 2 to 4). Eight-week-old pigs were fasted overnight. General anesthesia was induced and maintained with halothane. A total of 6 to 14 ligated intestinal loops were created in each pig by a method previously described (15). Each loop was 10 cm long; interloops were 2 to 4 cm long. The first loop was created 100 cm distal to the ligament of Treitz, and subsequent loops were created distally from the first. The location of a particular inoculum was varied from pig to pig, so that a particular inoculum did not always occur at one location within the intestine.
neutrophils are not present in section; +, neutrophils are occasionally present in villi; ++, neutrophils are seen consistently in subepithelial capillaries, within the epithelial cell layer, and within the lamina propria of the villi; ++++, neutrophils are plentiful in the above areas, and the epithelium at villous tips is occasionally absent; ++++, criteria for (++++ ) are present, and the epithelium at villous tips is consistently absent—a crust of neutrophils on the villi is at least 15 neutrophils thick.

Duplicate sections of ligated loops from experiment 2 were stained with a Brown-Hopps modified Gram stain (21) to facilitate microscopic evaluation of layers of bacteria adherent to intestinal epithelium. Adherence was determined as the presence or absence of bacterial layers on the villi.

Evaluation of numbers of bacteria (experiment 3). To evaluate the effect of bacterial proliferation on the alteration of E. coli to elicit neutrophils into the lumina, seven ligated loops were created in each of eight 8-week-old pigs. Each loop was inoculated with $2.5 \times 10^7$ bacteria (one loop for each of the first six E. coli strains shown in Table 2) or with TSB. The different strains were rotated in the different pigs in terms of location within the intestine. Four pigs were allowed to survive for 4 h after intestinal loop surgery; four were allowed to survive for 6 h.

At the time of necropsy, each loop was collected aseptically. The tissue and fluid from each loop were homogenized with a Virtis 45 homogenizer (The VirTis Co., Inc., Gardiner, N.Y.), and the volume of the homogenate was recorded. The concentration of viable bacteria in each homogenate was determined by using a spiral plater (Spiral Systems, Inc., Cincinnati, Ohio).

The different loops were compared in terms of the number of bacteria per milliliter and the total number of bacteria per loop.

STb production (experiment 4). Two strains of E. coli were obtained from S. L. Moseley. One strain, E. coli HB101, was nonenterotoxigenic. The other strain, E. coli HB101(pRAS-1), differed from the first strain only in that it contained a recombinant plasmid with the cloned gene for STb (13). These two strains were inoculated separately in 10 ml of BHl and incubated overnight at 37°C. Only 0.5 ml of this material was used to inoculate 400 ml of BHl in a 2,000-ml Erlenmeyer flask. This flask was incubated for 20 h at 37°C while being agitated on a platform shaker at approximately 240 movements per hour. Supernatant was prepared as described above.

STb loops and samples (experiment 4). In each of six 8-week-old pigs, six ligated intestinal loops were created. Two loops were exposed to BHI, two others were exposed to supernatant from strain HB101, and the last two were exposed to supernatant from strain HB101(pRAS-1) (STb). The pigs were killed and necropsied 4 h after surgery.

The volume of fluid in each loop was recorded. Smears of this fluid were fixed and stained, and the percentage of cells that were neutrophils was determined. Tissues from three loops from each pig (one loop per treatment) were fixed as described above and were then graded for the presence of neutrophils as described above.

Statistical methods. Levels of significance were determined by the Student t test (22).

RESULTS

Intact neonatal piglets (experiment 1). Strain 123 did not colonize the small intestine (form layers of bacteria adherent to villi), and the number of neutrophils in the mucosae and

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>No. colonized/total no.*</th>
<th>Histological grading of neutrophils in intestinal wall</th>
<th>Mean % neutrophils among host cells in smears of luminal fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0/12</td>
<td>0/12</td>
<td>2.0 (8)</td>
</tr>
<tr>
<td>123</td>
<td>0/6</td>
<td>1/6</td>
<td>0.3 (4)</td>
</tr>
<tr>
<td>431</td>
<td>4/6</td>
<td>6/6</td>
<td>25.8 (4)</td>
</tr>
<tr>
<td>987</td>
<td>5/6</td>
<td>5/6</td>
<td>36.5 (4)</td>
</tr>
</tbody>
</table>

* Data represent piglets necropsied at 8 and 16 h postinoculation.

** Number of samples with layers of bacteria adhered to villi. There were three samples per pig: upper jejunum, lower jejunum, and ileum. There were four control pigs and two pigs each for other treatments. Negative colonization values for strains 431 and 987 reflect noncolonization of the upper jejunum.

** Histological sections graded + or greater/number of sections examined.

The one negative value for strain 987 was an upper jejunal sample.

** Number in parentheses is the number of smears examined.

the percentage of neutrophils in the lumina of the intestines of pigs inoculated with strain 123 did not differ from those of the controls. The other two strains (431 and 987) began to colonize the small intestine at 2 and 8 h postinoculation, respectively. Colonization was correlated with increased numbers of neutrophils in the intestinal mucosa and an increased percentage of neutrophils among cells in the lumen (Table 3).

In pigs which had visible adherent bacterial layers, neutrophils were clearly present in the mucosa of the small intestine (especially in the lower small intestine). They were consistently seen in the subepithelial capillaries, between epithelial cells, and in the intestinal lumen. The lumen contained knots of neutrophils and bacteria. Occasional small gaps in the epithelium were noted on the distal one-third of villi, and sometimes neutrophils appeared to be entering the lumen through such gaps.

In the intact neonatal piglets, we did not have a method for measuring the total neutrophils in the lumen of the gut. We tried to evaluate the total number of cells elaborated into the different guts on a relative basis by examining the total number of cells in the luminal smears. The smears which had a higher percentage of neutrophils also had markedly more total cells. Therefore, we concluded that an increase in the percentage of neutrophils in smears of luminal fluid reflected an absolute increase in the total number of neutrophils in luminal fluid.

Neutrophils attracted by different strains of E. coli in ligated intestinal loops (experiment 2). Strains such as 123, which do not colonize intact pigs, can attain high numbers in ligated intestinal loops even though they do not adhere to villi. Intestinal loops also permit comparisons among several strains in the same animal. Each of the first six strains (Table 2) was tested at least once in ligated intestinal loops in each of six different pigs. Data from 4- and 6-h incubations were pooled to measure neutrophil response to a strain, because these data were of a similar magnitude. Data from the 2-h incubation were not included in this pool because responses at 2 h were uniformly minimal.

Different strains of E. coli were inoculated in a total of two to four replicate loops for 6 h of incubation and three to five replicate loops for 4 h of incubation. Loops containing only TSB and mannitol were replicated four times for 6 h of incubation and eight times for 4 h of incubation.

The first data to be compared were those of the percentage
of neutrophils among cells in smears of loop fluid. Figure 1 shows these means and their standard errors. When these means were compared, strains 263 and 1288 clearly attracted more neutrophils than did the control (P < 0.01). Values for the other strains did not differ from those for the controls.

The histological rankings for each strain were averaged and are also shown in Fig. 1. Strains 263 and 1288 again differed from the controls (P < 0.01) (Fig. 1). The remaining strains did not differ from the controls.

When averages of total neutrophils per loop were compared by strain, strains 263, 987, and 1288 were found to be significantly different from the controls (P < 0.01) (Fig. 1). The remaining strains did not differ from the controls.

We also summarized data to examine changes over time for each strain. Only strains 263, 987, and 1288 yielded a significantly greater percentage of neutrophils after 6 h of incubation than they did after 4 h of incubation (P < 0.05).

Adherence of bacteria in loops (experiment 2). We considered the possibility that bacterial adhesion was required for a strain to elicit neutrophils. For example, strain 431 caused a positive response in intact neonates where it adhered readily. It does not adhere well in vitro to cells from pigs of the age used for the ligated intestinal loop studies (18) and also did not attract neutrophils in these older pigs. For this reason, we reviewed Gram-stained histological sections from all the intestinal loops and scored them by using layers of bacteria associated with villous epithelium as an index of adhesion (5). There was no correlation between the occurrence of epithelial-associated bacteria and neutrophil response.

Comparison of live and killed E. coli cells (experiment 2). Five different methods of killing E. coli 263 were used. All methods successfully killed the bacteria and all elicited similar numbers of neutrophils into ligated loops. Therefore, these data were pooled and referred to by "killed 263." The number of neutrophils elicited into a loop by killed 263 did not differ significantly from control values (Table 4). However, the number of neutrophils elicited into a loop by live E. coli 263 did differ from control values (P < 0.01).

Growth of bacteria in loops (experiment 3). The mean number of E. coli cells per loop after 4 and 6 h of incubation for all six strains ranged from 2 × 10⁸ to 8 × 10⁸. The three strains of E. coli which attracted the most neutrophils into loops (263, 1288, and 987) in experiment 2 were not present in higher numbers than were the other three strains.

Chemotaxis and STb (experiment 4). The three strains of E. coli which attracted the largest numbers of neutrophils into the lumen in ligated intestinal loops shared one factor which the other strains did not: an ability to produce STb (Table 2; Fig. 1). This contrasted with the results in intact neonatal piglets, in which strains 431 (heat-stable toxin a [STa] only) and 987 (STa and STb) both attracted neutrophils. However, we wanted to determine whether STb could be one factor which attracts neutrophils. For that reason, we obtained the E. coli strains HB101 and HB101(pRAS-1) and tested the ability of sterile culture supernatants of these strains (STb preparations) to attract neutrophils into ligated intestinal loops.

Four of the six pigs tested responded to STb enterotoxin, as noted by the presence of larger amounts of fluid in these loops after 4 h of incubation (P < 0.01) (mean ± standard error was 10.6 ml ± 1.15 for the E. coli HB101 loops and 31.2 ml ± 2.24 for the E. coli HB101(pRAS-1) loops). In these four pigs, there was no relationship between neutrophil attraction into the lumen or wall of the intestine and the presence or absence of STb in a ligated intestinal loop (mean ± standard error for percentage of neutrophils was 18.9 ± 9.2 for BHI loops, 26.9 ± 9.2 for E. coli HB101 loops, and 31.0 ± 9.7 for E. coli HB101(pRAS-1) loops).

<table>
<thead>
<tr>
<th>Loop contents</th>
<th>n</th>
<th>Mean no. of neutrophils/loop ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>79,730 ± 58,507*</td>
</tr>
<tr>
<td>Killed 263</td>
<td>5</td>
<td>260,049 ± 86,718*</td>
</tr>
<tr>
<td>Live 263</td>
<td>3</td>
<td>3,357,082 ± 393,024*</td>
</tr>
</tbody>
</table>

* Means with different superscripts are different, P < 0.01.
DISCUSSION

We demonstrated that neutrophils entered the lumen of the small intestine of neonatal piglets when that intestine was intensively colonized with ETEC and that exposure to a nonenteropathogenic strain of E. coli did not attract similar numbers of enteroluminal neutrophils. Neutrophils with phagocytosed E. coli cells have been reported on the villi of neonatal piglets with colibacillosis (14). Jubb and Kennedy also report a purulent intestinal content, which is occasionally observed in piglets with colibacillosis (10). However, this is the first experimental demonstration that when the small intestine of a neonatal piglet is colonized with E. coli cells (at least with these two strains), enteroluminal neutrophils are seen in significant numbers.

Although the small intestine of intact neonatal piglets is the most natural system in which to study this phenomenon, the ligated intestinal loop is better suited for dissecting out variables which could influence the attraction of neutrophils. Gut motility is not a factor when one is working with ligated loops, and it is possible to compare several different treatments within one animal.

It seems unlikely that there is any single bacterial attribute which determines elicitation of neutrophils. Many types of agents have been demonstrated to affect neutrophil behavior; these range from formyl-methionyl peptides to lipopolysaccharides to immune complexes (17). The aim of our work with ligated loops was to attempt to identify one or some of the factor(s) which might attract neutrophils into the lumen of the intestine.

The ability to attract neutrophils into the lumina of ligated intestinal loops varied with the strain of E. coli. Strains 263, 1288, and 987 definitely attracted more neutrophils into the intestinal lumina than did the controls. These same three strains continued to attract neutrophils at 6 h postinoculation, when other strains did not.

The three strains which elicited the strongest luminal neutrophil response in the ligated loops (strains 263, 1288, and 987) share one factor which the other strains do not (Table 2). This factor is STb. According to Bellamy and Nielsen (3), neutrophils could be elicited into the lumen if antigen was placed in the lumen and if the animal in question had specific circulating antibody. Possibly our pigs, which were all obtained from the same source, shared a similar history of antigen exposure. However, this work spanned 5 months, and it seems unlikely that this herd sustained continued exposure to STb but not to other antigens of E. coli. Furthermore, STb has minimal antigenicity (22). Thus, immunity to STb seems unlikely to explain the neutrophil response to these strains.

The STb could conceivably attract neutrophils either directly or even indirectly, through subtle tissue damage. However, examination of porcine ligated intestinal loops exposed to supernatants of E. coli HB101 and HB101(pRAS-1) indicated that STb had no effect on the number of neutrophils attracted to the wall or lumen of the intestine. (Fluid secretion was comparable in these supernatant loops and in loops containing whole STb+ bacteria; STb concentrations in these loops were also judged to be comparable.) Furthermore, strain 431 (which does not produce STb) elicited neutrophils in intact neonatal piglets (Table 3). Thus, the enteroluminal neutrophil response to porcine ETEC cannot be explained by STb per se.

Two of the strains which attracted neutrophils so strongly (strains 263 and 1288) are labile toxin (LT) positive. We did not test for the role of LT in the attraction of enteroluminal neutrophils in loops. However, in vitro evidence indicates that LT suppresses chemotactic activity of neutrophils (4).

We also considered the possibility that bacterial adhesion to epithelium might be required, in view of the fact that strain 431 gave a positive response in neonates, in which it adheres, but was negative in older pigs, in which it does not adhere (18), and that the 987P and K88 antigens of the two strains that gave positive reactions in intestinal loops of older pigs mediate adhesion to pig intestine. We were unable to demonstrate any correlation between positive response and adhesion by examining histological sections from the loops. This could indicate that adhesion is not required for a neutrophil response in the closed loop system, where bacteria can attain high numbers without adhesion, or it could indicate that the histological method used was not appropriate for differentiating between adherent and nonadherent bacteria. The role of adhesion in attracting neutrophils could perhaps be determined by using isogenic pairs (with and without the adhesins) of strains that do attract neutrophils.

The E. coli cells apparently had to be alive to attract neutrophils into the intestinal lumen. However, the strains which attracted more neutrophils into the lumina of loops did not achieve this by an exceptional proliferation of E. coli cells; strains which did not attract neutrophils grew just as rapidly as those that did.

How do the numbers of neutrophils we observed compare with numbers of neutrophils noted in other conditions or sites? If we examine the neutrophils elicited into ligated intestinal loops by E. coli 263 after 6 h of incubation, we find the gut fluid contains 460 neutrophils per μl. A slightly younger pig has approximately 5,400 neutrophils per μl in the blood (19). Bellamy and Nielsen were able to attract nearly four times as many neutrophils into intestinal loops as we were (3). The mammary gland is another mucosal surface on which neutrophils are known to be present in large numbers. Normal cows in the latter part of lactation have neutrophil counts as high as 1,000/μl (6). Neutrophil counts in excess of 3,600/μl occur in milk from infected glands (7, 8). Because all these measurements represent static measurements of dynamic neutrophil populations, they may not mean as much as we would like them to. That is, a study of gut neutrophil kinetics would have more meaning than does this type of measurement (16).

In response to Sellwood’s question, we have shown that neutrophils are present in increased numbers in the intestinal lumina and walls of neonatal piglets suffering from colibacillosis. Using the ligated intestinal loop model in older pigs, we found that attraction of neutrophils into the intestine was influenced by the strain of E. coli inoculated into the loops. At this point, we have found no single attribute of an E. coli strain which is correlated with its ability to attract neutrophils. In the future, we hope to elucidate the abilities these enteroluminal neutrophils have with respect to bacterial killing.

ACKNOWLEDGMENTS

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LITERATURE CITED