NOTES

Small-Bowel Colonization Alone Is a Cause of Diarrhea

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Colonization of the small bowel is the usual first step in the pathogenesis of acute infectious diarrheal disease. Furthermore, bacterial overgrowth in the small bowel is known to occur in association with many types of diarrhea (1, 2, 5, 7, 8, 20), malnutrition (9, 11, 15, 17), and intestinal stasis. However, the role of bacterial overgrowth or colonization alone in causing small-bowel fluid secretion has been unclear. Classic studies of enterotoxigenic Escherichia coli in piglets and, more recently, in humans raise the possibility that specific adherence traits, in the absence of recognized toxin production, may cause diarrhea (14, 18). Data from strains of E. coli with the adherence trait K88 suggest an association between colonization with organisms with this trait and diarrhea in piglets (18). In addition, E. coli vaccine trials in human volunteers with colonizing but nonotoxigenic organisms were associated with diarrhea in 2 of 19 volunteers (14). However, a true causal relationship between colonization and fluid production has not been clear, nor have the potential mechanisms of fluid production been elucidated.

To examine whether colonization alone can cause small-bowel fluid secretion or diarrhea in a reproducible model with organisms derived from human pathogens, we studied human colonizing factor antigen (CFA)-positive E. coli in a rabbit model. We found that colonization occurs and is associated with significant fluid secretion in the small bowel and diarrhea at 72 but not 18 h in this model.

E. coli 1392* (O6:H16) with CFA/II and 1392* without CFA/II were kindly provided by the Center for Vaccine Development, University of Maryland. These strains are spontaneous laboratory derivatives of E. coli 1392 which previously contained the plasmid for both heat-labile and heat-stable enterotoxins (14). Assays of these strains in our laboratory for heat-labile toxin by CHO cell stretching (10), for heat-stable toxin by the suckling mouse assay (4), for cytotoxicity of culture filtrates on Vero, CHO, and HeLa cell lines, and for distinctive adherence to the HEP2 cell line (3) were all negative. In addition, DNA hybridization probes for both human and porcine heat-stable toxin and heat-labile toxin were negative (13).

Strains were maintained on nutrient agar and, when used in assays, were grown in LT broth (Casamino Acids, yeast extract, and glucose) for 18 h with shaking. When broth cultures were used, this 18-h growth was adjusted to a McFarland no. 8 standard (2.4 × 109 organisms per ml). When culture filtrates were used, broth cultures were centrifuged at 4°C and 10,000 × g for 25 min, and supernatants were filtered through a 0.22-μm-pore filter (Millipore Corp., Bedford, Mass.) and used immediately. The presence of CFA/II in strains was determined by hemagglutination with bovine erythrocytes (Hazleton/Dutchland Inc., Denver, Pa.) as previously described (6).

The 18-h rabbit ligated loop model was used to study colonization and secretion. Segments (4 to 6 cm) were made in the ilea of 2-kg New Zealand White rabbits anesthetized with ketamine (Parke, Davis & Co., Morris Plains, N.J.) and xylazine (Miles Laboratories, Inc., Shawnee, Kans.) as previously described (16). Culture filtrates (1 ml) or a whole bacterial culture of test strain at a McFarland no. 8 standard (1 ml) was used in each doubly ligated loop.

A modification of the reversible ileal tie model, as first described by Spira et al. (19), was used to study intestinal colonization beyond 18 h. Animals were studied simultaneously in pairs with strains 1392* and 1392*. When 1.5- to 2-kg New Zealand White rabbits were under ketamine-xylazine anesthesia, a removable slip knot was placed around the mid-ileum. A whole bacterial culture at a McFarland no. 8 standard (10 ml) was used as the inoculum for the test strains, and 10 ml of LT broth was used in control animals. Inoculation was intraluminal, 10 cm proximal to the reversible tie. The abdominal incision was closed after intraluminal inoculation. At 4 h, the slip knot was removed, leaving the gut patent. The animals were allowed to eat and drink normally while being monitored clinically.

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TABLE 1. Results in rabbits 72 h after inoculation of $10^{10}$ CFU of organisms or of LT broth (control) 10 cm proximal to the reversible ileal tie

<table>
<thead>
<tr>
<th>Inoculating strain (no. of rabbits)</th>
<th>No. of rabbits with (+) or without (−) diarrhea (mean CFU/cm²)</th>
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<tbody>
<tr>
<td>1392⁺ (11)</td>
<td>7⁺ ($10^{10.4} ± 1.6b$)</td>
</tr>
<tr>
<td>1392⁻ (10)</td>
<td>0⁻ ($10^{10.6} ± 1.0b$)</td>
</tr>
<tr>
<td>None (control) (4)</td>
<td>0⁻ ($10^{9.8} ± 0.2b$)</td>
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*a* $P < 0.005.$
*b* $P < 0.05.$

Quantitative cultures were done by excising 1 cm² of mucosal tissue and vortexing it in 1 ml of phosphate-buffered saline. Serial dilutions were made, and 0.1-ml aliquots were cultured on MacConkey and blood agar plates.

Specimens were processed promptly for histology by fixation in Formalin. After the specimens were embedded in paraffin, both hematoxylin-eosin staining and tissue Gram staining were done.

Statistics were analyzed by Student’s $t$ test and by chi-square analysis. Data are expressed as the mean ± standard deviation of the mean.

In the 18-h ligated ileal loop model, the positive controls (cholera toxoid and both a whole culture and a culture filtrate of *E. coli* B16-4, which has both heat-labile and heat-stable toxins) were positive, with a fluid volume/length ratio (milliliters/centimeter) of more than one. The negative controls (whole broth cultures of nonpathogenic *E. coli* K-12) produced no fluid. Inoculation of either a whole culture or a culture filtrate of either test strain, 1392⁺ or 1392⁻, did not produce positive fluid volume/length ratios in ileal loops (mean, <0.15) at 18 h. Quantitative aerobic and anaerobic cultures of 1 cm² of tissue were done to determine if colonization was taking place. Anaerobic cultures revealed only gram-negative rods confirmed as *E. coli* by the API system (Analytab Products, Sherwood, N.Y.), and subsequently only aerobic cultures were done. Strain 1392⁺ colonized loops at a mean of $10^{2.1}$ CFU/cm², representing nearly 1,000-fold more CFU/cm² than the values for strain 1392⁻ ($10^{6.2}$ CFU/cm²) or for loops inoculated with LT broth alone ($P < 0.05$). The organisms recovered were pure gram-negative rods. The organisms recovered from the loops inoculated with strain 1392⁻ were positive in the bovine hemagglutination assay for CFA/II. The organisms recovered from the loops inoculated with strain 1392⁺ or LT broth did not agglutinate either bovine or human type A erythrocytes, indicating the absence of CFA/II. Light microscopy of tissue from loops inoculated with either 1392⁺ or 1392⁻ showed no mucosal abnormality.

The reversible ileal tie model results at 18 h were comparable to the ligated ileal loop model results at 18 h. Colonization occurred at a similar level (means, $10^{9.4}$ CFU/cm² in ileal sections in the reversible ileal tie model and $10^{9.1}$ CFU/cm² ligated loops for strain 1392⁺ and $10^{8.2}$ CFU/cm² in ileal sections in the reversible ileal tie model and $10^{8.2}$ CFU/cm² in ligated loops for strain 1392⁻). Results of quantitative cultures done at various points along the small-bowel varied from a low of $10^{8.5}$ CFU/cm² in the proximal ileum to consistently higher values in the mid-ileum and distal ileum ($10^{9.4}$ and $10^{9.3}$ CFU/cm², respectively, for strain 1392⁻) and mid-ileum cultures were thereafter used for comparison. No gut fluid or diarrhea was seen. To determine if colonization over a longer period of time altered the outcome, we extended the observation period after removal of the tie to 72 h.

Results of the 72-h reversible ileal tie model are seen in Table 1. In these experiments, frank diarrhea, defined as the occurrence of wet, brown, matted fur in the anal area, developed in 7 of 11 animals inoculated with strain 1392⁺ ($P < 0.005$). The range of organisms recovered in quantitative cultures of 1 cm² of mucosa is shown in Fig. 1. Mean colonization in rabbits with diarrhea was $10^{10.4}$ CFU/cm². All seven animals with diarrhea had more than $10^{8.5}$ CFU/cm² of mucosa. No animal inoculated with strain 1392⁻ had more than $10^{8.0}$ CFU/cm² of mucosa ($P < 0.05$). No animal with less than $10^{6.8}$ CFU/cm² of mucosa, whether 1392⁺ or 1392⁻, developed diarrhea. Organisms recovered from animals that were given strain 1392⁻ and that developed diarrhea did not produce fluid when they were reinoculated in 18-hour ligated ileal loops.

To assess how tightly adherent the bacteria were to the gut mucosa, we lightly washed 1 cm² of tissue from an animal colonized with strain 1392⁺ in sterile phosphate-buffered saline. The original tissue had $10^{6.6}$ CFU/cm² and the washed mucosa from the same animal retained less than $10^{5.0}$ CFU/cm². The phosphate-buffered saline wash solution contained 10⁻¹ CFU/cm². These results suggest that the organisms were loosely adherent to the mucosa.

Histologic studies of ileal and jejunal tissues obtained from diarrheic rabbits colonized for 72 h with strain 1392⁺ revealed no mucosal abnormalities under light microscopy. Organisms were only rarely seen on the mucosa.

An analysis of small-bowel fluid from four diarrheic rabbits colonized with strain 1392⁺ revealed a mean protein level of 0.6 ± 0.02 g/dl; the mean normal rabbit serum protein level (from control animals) was 6.1 ± 0.4 g/dl. The mean osmolality of the small-bowel fluid from these four animals was 281 ± 7 mosmol/liter; the mean normal rabbit serum osmolality (from control animals) was 305 ± 5 mosmol/liter.

At 18 h in either model, an *E. coli* strain with CFA/II but without a known enterotoxin colonized rabbit mucosa without producing fluid. Of note, both strains showed equivalent growth in vitro in LT broth; therefore, the different degrees of

FIG. 1. Range of quantitative culture results from the 72-h reversible ileal tie model. Of 11 animals given strain 1392⁺, 7 were colonized with more than $10^{6.4}$ CFU/cm² and developed diarrhea. Of 11 animals given strain 1392⁻, 4 were not colonized to this extent and did not develop diarrhea. None of the 10 animals given 1392⁻ and none of the control animals were colonized with more than $10^{8.0}$ CFU/cm² or developed diarrhea.
of colonization should not be the result of different degrees of growth.

When the reversible ileal tie model was extended to 72 h, the degree of colonization in the animals given strain 1392± continued to increase and was significantly higher than in those given strain 1392−. Furthermore, animals that were colonized with at least 10^8 CFU/cm² consistently developed diarrhea. The facts that four animals given strain 1392+ were not colonized to the same extent as were animals that developed diarrhea and that these four animals did not develop diarrhea may well be explained either by interanimal variability or a variation in individual rabbit mucosal immunity. Differences between rabbit and human epithelial receptors may also contribute to variability in colonization.

We suspect that bacterial adherence is loose in this rabbit model, since organisms were so readily removed from the mucosa by a light wash. While the receptor for CFA/II on human gut tissue has not yet been identified, the loose adherence of the organisms may also be related to variations in receptors between species. An alternative explanation may be that the organism binds to intestinal mucus rather than to gut epithelium (12). Bacterial binding to mucus could also explain why organisms were not readily visible on tissue sections fixed in Formalin.

The nearly iso-osmotic nature of the nonproteinaceous small-bowel fluid produced in these rabbits suggests but does not prove a secretory mechanism for the diarrhea.

In summary, our studies demonstrate that small-bowel colonization by a nontoxigenic E. coli strain with CFA/II is consistently associated with the production of fluid in the small bowel and with diarrhea. However, the mechanism of fluid production is unknown. Several possibilities exist, including the possibility that the bacteria produce an as-yet-unrecognized toxin which requires a prolonged delivery to the mucosa or brush border to exert its effects or which is present in such small amounts that its effects are seen only when bacteria are present in large numbers. Conversely, the mechanism of fluid production may be via the metabolic or enzymatic products of the bacteria, acting either directly in the gut as secretagogues or as agents capable of altering gut absorption or gut motility by neurohumoral mechanisms.

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LITERATURE CITED


