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Net Fluid Secretion and Impaired Villous Function Induced by Colonization of the Small Intestine by Nontoxigenic Colonizing *Escherichia coli*

THERESA A. SCHLAGER, CHRISTINE A. WANKE, AND RICHARD L. GUERRANT*

Division of Geographic Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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The role of colonizing bacteria in the small bowel in causing diarrhea remains unclear. We examined whether colonizing, nontoxigenic *Escherichia coli* could alter small bowel function by determining net water and electrolyte fluxes and sucrase activity in colonized and noncolonized ileal segments by using the reversible-ileal-tie adult rabbit model. Colonization of the ileum with nontoxigenic *E. coli* for ≥72 h at ≥10^4/cm^2 was associated with significant functional derangements, as follows: (i) overt liquid diarrhea in 50% of animals colonized at >10^4/cm^2; (ii) reversal of normal net ileal absorption to net secretion of water, sodium, and chloride; and (iii) significant decrease in mucosal sucrase activity. We conclude that small bowel colonization by colonizing, nontoxigenic *E. coli* impairs water and electrolyte absorption and sucrase activity in the absence of recognized enterotoxin, cytotoxin, invasion, or effacement traits.

Diarrheal diseases constitute a leading cause of death on a global scale among young children, accounting for an estimated 4.6 million deaths each year (>12,600 deaths per day) in Asia, Africa, and Latin America (32, 36). Although infectious diarrheas are usually benign and self-limited in industrialized countries, they remain a major cause of morbidity and account for 3 to 5% of pediatric hospital admissions (26). With the development and application of oral rehydration therapy at the community level and the consequent reduction in acute diarrhea mortality, prolonged diarrheal illnesses are emerging as the major cause of diarrhea mortality. In a study (using cemetery surveillance and "verbal autopsies," family interviews after recent childhood deaths) by McAuliffe et al. in Guaiuba, a rural town near Fortaleza, Brazil, fully one-half of childhood deaths due to diarrhea were due to prolonged illness rather than to acute illness. Furthermore, a prolonged diarrheal illness consistently identifies children at high risk for heavy diarrhea burdens and often with accompanying severe malnutrition (1, 20, 29).

We and others have noted the frequent association of small intestinal bacterial colonization in infants and children with prolonged diarrheal illness and malnutrition (2, 8, 9, 12, 19, 25). However, the role of colonizing bacteria in the small bowel in causing diarrhea or altering small bowel function remains unclear. Pioneering studies by Smith and Linggood demonstrated that, while both a colonization trait (K88) and enterotoxin were necessary for *Escherichia coli* to elicit acute diarrhea in most piglets, nontoxigenic *E. coli* with K88 alone caused diarrhea in about one-third of experimentally infected piglets (31). Vaccine trials with colonizing but nontoxigenic *E. coli* given in doses of 10^9 to 10^{10} CFU/ml caused diarrhea in 2 of 19 human volunteers (15). In addition, colonizing, nontoxigenic *Vibrio cholerae* given at 10^4 to 10^{10} CFU/ml to human volunteers resulted in protective immunity to experimental challenge, but over half the volunteers experienced mild diarrhea (17). Although the use of a vaccine strain that did not produce Shiga-like toxin or El Tor hemolysin reduced diarrhea rates, mild diarrhea still occurred in 12% of volunteers given 10^6 to 10^8 CFU of vaccine strain CVD 103 per ml (16). Wanke and Guerrant, in the reversible-ileal-tie adult rabbit (RITARD) model, demonstrated that small bowel colonization by nontoxigenic *E. coli* was consistently associated with small bowel fluid secretion and diarrhea after 3 days (37). These findings were confirmed by Sack et al., who showed that colonizing, nontoxigenic *E. coli* at >10^9 CFU/ml given to 16 rabbits by using the RITARD model caused diarrhea in 8 animals and lethal diarrhea in 6 animals (27).

The present study was undertaken to further examine the alteration of small bowel physiology by bacterial colonization in the rabbit model. We focused on water and electrolyte transport and enzyme alterations in the presence of small bowel colonization with nontoxigenic *E. coli*.

MATERIALS AND METHODS

Bacterial strains. *E. coli* 1392+ (O6:H16, identified and fully characterized as strain E1392/75-2A [15] with colonization factor antigen II [CFA/II] [CS1 and CS3]) and 1392– without CFA/II were kindly provided by James B. Kaper at the Center for Vaccine Development, University of Maryland, Baltimore. These strains are spontaneous laboratory derivatives of *E. coli* 1392, originally from the laboratory of Bernard Rowe, Central Public Health Laboratory, Colindale, England, from a patient with acute diarrhea, a strain which previously contained the plasmid for both heat-labile and heat-stable enterotoxins (15). *E. coli* 132-3 (O75ac:H7), a nontoxigenic strain which did not express CFA/II, was isolated from the stool of a child in Brazil with prolonged diarrhea and was chosen in order to study the alterations of small bowel function upon colonization by a strain without recognized colonization, enterotoxin, cytotoxin, or effacement traits. The pMAR gene probes for localized adherence (22) or for the PCFO159:H4 adhesin, as described by Tacket et al. (34), were all negative (kindly tested by Kaye Wachsmuth, Centers for Disease Control, Atlanta, Ga., and James B. Kaper, University of Maryland, Baltimore). Strain 132-3 is highly hydrophobic and has numerous 6- to 8-nm surface fimbriae. Assays of 1392+, 1392−, and 132-3 in our laboratory for heat-labile toxin by CHO cell elongation (10),

* Corresponding author.

for heat-stable toxin by the suckling mouse assay (5), for cytotoxicity of culture filtrates on Vero, CHO, and HeLa cell lines, and for distinctive adherence to the HEp-2 cell line (3) were all negative.

Bacterial inoculum. Strains were cultured on CFA agar for 24 h before use (7). The colonies were scraped from the agar plates and suspended in CFA broth. The presence of CFA/II was confirmed in strain 1392+ when it was inoculated and again when it was reisolated as the colonizing organism at 72 h by hemagglutination with bovine erythrocytes (Hazelton/Dutchland Inc., Denver, Pa.) (6). Similarly, the hydrophobicity of *E. coli* 132-2 was confirmed by precipitation in 0.03 M $(NH_4)_2SO_4$ when it was inoculated and again when it was reisolated as the colonizing organism at 72 h (18).

Animal model. A modified version of the reversible-ileal-tie model, first described by Spira et al. (33), was used to study intestinal colonization at 72 h. New Zealand White rabbits (1.5 to 2 kg each) were fasted for 24 h and then anesthetized with ketamine-xylazine, and the cecum of each was tied off. A full-thickness biopsy was taken in the ileum 6 cm proximal to the appendix for sucrase determination, the ileum was repaired with silk suture, and then a removable slipknot was placed within 5 cm proximal to the biopsy. Fifteen milliliters of a whole bacterial culture, McFarland no. 8 (confirmed by quantitative counts to be approximately 2.4×10^9 organisms per ml), was used as the inoculum for the test strains. Control rabbits were inoculated with a similar bacterial inoculum, but of the noncolonizing 1392^- strain, and others were not inoculated. Bacteria were inoculated intraluminally within 5 cm proximal to the removable slipknot. The abdominal incision was closed after intraluminal inoculation. After 4 h, the slipknot was removed, leaving the gut patent. The animals were allowed to eat and drink ad libitum while being monitored clinically each day.

At 72 h, the animals were again placed under ketamine-xylazine anesthesia after being fasted for 24 h. Within 5 cm proximal to the tie location, a 1-cm^2 full-thickness biopsy was taken for quantitative cultures, as was a second full-thickness biopsy for sucrase determination and electron microscopy. The ileum was repaired with silk suture, and then two 9-cm loops were ligated in the distal ileum and 4 ml of nonabsorbable volume marker, phenolsulfonphthalein (PSP), 5 mg/100 ml, was placed in each loop and mixed; 1 ml was removed at time zero from each loop, and the remainder was removed at 4 h. The animals were then sacrificed.

Quantitative cultures. Quantitative cultures were prepared by excising 1 cm^2 of mucosal tissue and vortexing it in 1 ml of phosphate-buffered saline. Serial dilutions were made, and 0.1-ml aliquots were cultured aerobically and anaerobically on MacConkey and blood agar plates. Anaerobic cultures failed to reveal $>10^2$ anaerobes, and subsequently only aerobic cultures were prepared. Cultured isolates from the small bowel were subsequently grown on CFA agar, and hemagglutination and hydrophobicity patterns were checked as described above.

Net fluid and electrolyte transport determinations. Fluid removed from the ileal loops was assayed within 4 h of removal. The PSP concentrations were measured colorimetrically after alkalinization, and net volume changes were calculated as previously described (11). Electrolyte concentrations (Na, Cl) were measured at time zero and at 4 h by flame photometry, and net sodium and chloride fluxes were calculated by using these concentrations and the calculated volume changes.

Disaccharidase determinations. Sucrase was chosen instead of lactase for disaccharidase determinations because lactase activity decreases substantially in the postnatal period in the rabbit (35). Preliminary lactase determinations confirmed the very low lactase activity in our animals. Biopsy tissue was immediately frozen at −20°C for determination of the brush border enzyme sucrase by using the standard method of Dahlqvist (4). Briefly, the room-temperature tissue homogenate was incubated with sucrose for a specified time period. The amount of glucose liberated was measured colorimetrically by using a glucose oxidase reagent. The specific activity of sucrase was calculated as micromoles of glucose formed per minute per milligram of protein homogenate.

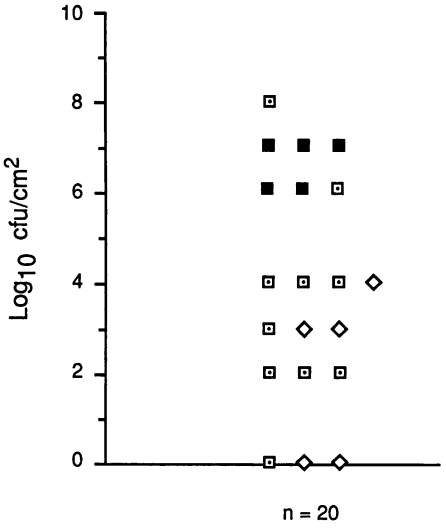

FIG. 1. Quantitative cultures at 72 h after inoculation of colonizing *E. coli* (strain 1392^+ or 132-3) (shown as squares) or of noncolonizing *E. coli* (strain 1392^-) or sham-operated controls (shown as diamonds). Of the five controls, four were sham-operated controls and one (with no growth) was inoculated with strain 1392. Closed squares indicate development of overt diarrhea at 24 to 72 h.

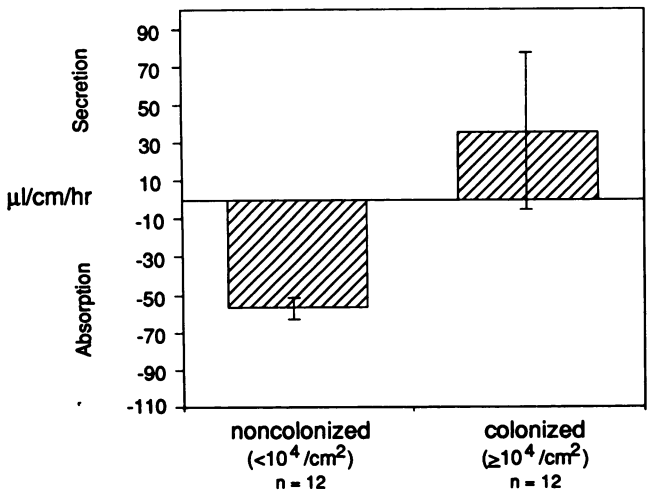

FIG. 2. Effect of small bowel colonization on net water transport as determined by calculations from PSP concentrations. The mean net absorption from noncolonized ileal segments is compared with the mean net secretion from colonized ileal segments ($P < 0.05$ by Student's t test). Error bars represent the standard error of the mean.

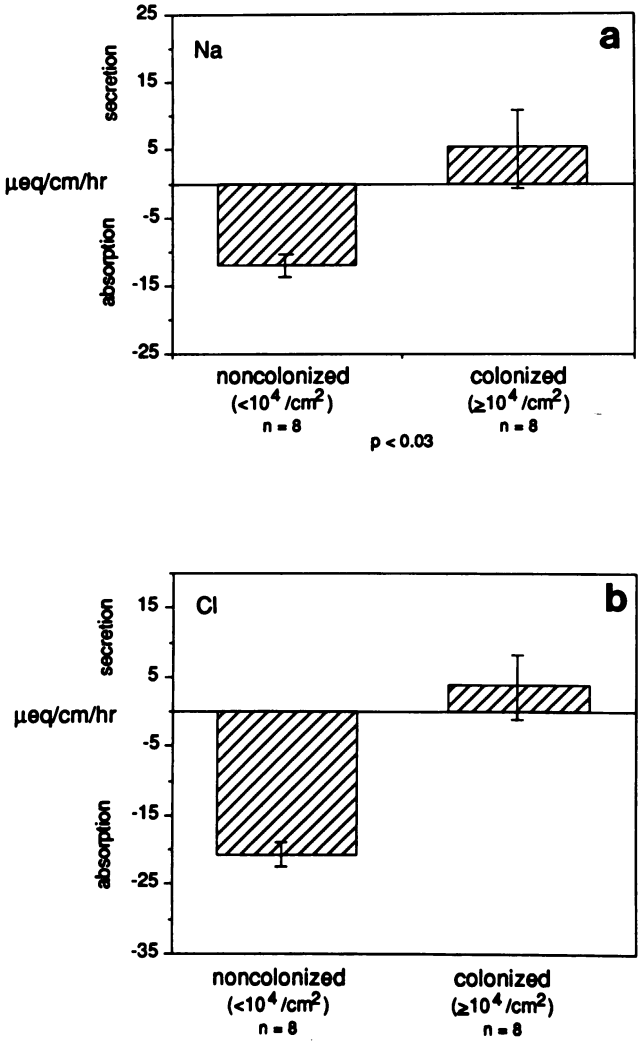

FIG. 3. Effect of small bowel colonization on net sodium (a) and chloride (b) transport. The mean net absorption from noncolonized ileal segments is compared with the mean net secretion from colonized ileal segments for sodium (a) and chloride (b) ($P < 0.03$ by Student's t test). Error bars represent the standard error of the mean.

Electron microscopy. Specimens for electron microscopy were placed in 2% glutaraldehyde before being processed at the Central Electron Microscope Facility at the University of Virginia, Charlottesville.

Statistics. Comparisons of data were analyzed by using the unpaired Student t test (except that the paired t test was used for the paired sucrase determinations in the same animal before and after colonization). Data are expressed as the mean ± standard error of the mean.

RESULTS

Twenty rabbits were studied. Fifteen rabbits were inoculated with *E. coli* 1392^+ ($n = 5$) or 132-3 ($n = 10$), and five rabbits either were inoculated with *E. coli* 1392^- or had sham operations but were not inoculated.

The results of quantitative cultures of vortexed mucosal tissues taken at 72 h are shown in Fig. 1. Five of the seven rabbits that were colonized at bacterium concentrations of greater than or equal to 10^6 CFU/cm^2 had diarrhea at 72 h.

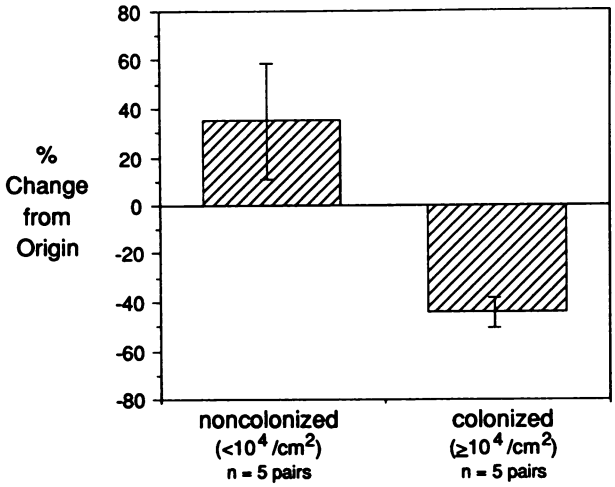

FIG. 4. Effect of small bowel colonization on mucosal sucrase activity. No consistent change from the original biopsy in noncolonized animals was seen. This result may be compared with the consistent and significant decrease in mean sucrase activity in colonized animals ($P < 0.03$ by Student's t test). Error bars represent the standard error of the mean.

The diarrhea produced was mild and was defined as a change in stool pellet consistency, identified on the basis of wet, brown, matted fur in the anal area, which occurred from 1 to 3 days after inoculation and continued to the time of sacrifice at 72 h.

We found that significant alterations in fluid and electrolyte absorption and sucrase activity occurred with inoculation of colonizing *E. coli* at concentrations at greater than or equal to 10^4 CFU/cm^2, and we analyzed all results by using $\geq 10^4$ CFU of colonizing or hydrophobic *E. coli* per cm^2 as our cutoff for colonization. One of the sham-operated rabbits (not inoculated) had 10^4 coliform organisms per cm^2; it did not exhibit any hydrophobicity or colonization traits by hemagglutination and was therefore included in the control group. This animal did not have any alteration in fluid or electrolyte transport or in disaccharidase activity.

The effects of small bowel colonization on net water transport are shown in Fig. 2. Studies of net water transport using the PSP volume marker were conducted with 24 rabbit ileal loops in 13 rabbits. These studies revealed consistent water absorption in the 8 control ileal loops and in the 4 loops that were colonized with less than 10^4 organisms per cm^2 (mean absorption, -56 μl of H_2O per cm per h). The 12 ileal loops that were colonized with greater than or equal to 10^4 colonizing *E. coli* per cm^2 showed a reversal of net absorption to mean net secretion (mean, +36 μl/cm per h; $P < 0.05$, $n = 24$ loops; $P < 0.03$, $n = 13$ rabbits).

The effects of small bowel colonization on net sodium (Na) and chloride (Cl) transport are shown in Fig. 3. Sodium and chloride measurements taken in 16 rabbit ileal loops in 10 rabbits (6 colonized and 4 uncolonized) revealed consistent absorption of sodium (mean = -12 μeq of Na per cm per h) and chloride (mean = $-$ 18.5 μEq of Cl per cm per h) in the 6 control loops and in the 2 loops that were colonized at less than 10^4 organisms cm^2. The 8 colonized loops showed a reversal of net absorption to net secretion of Na (mean = +5.5 μeq of Na per cm per h; $P < 0.03$, $n = 16$ loops; $P < 0.01$, $n = 10$ rabbits) and Cl (mean = +3.8 μeq of Cl per cm per h; $P < 0.03$, $n = 16$ loops; $P < 0.05$, $n = 10$ rabbits).

The effects of small bowel colonization on mucosal su-

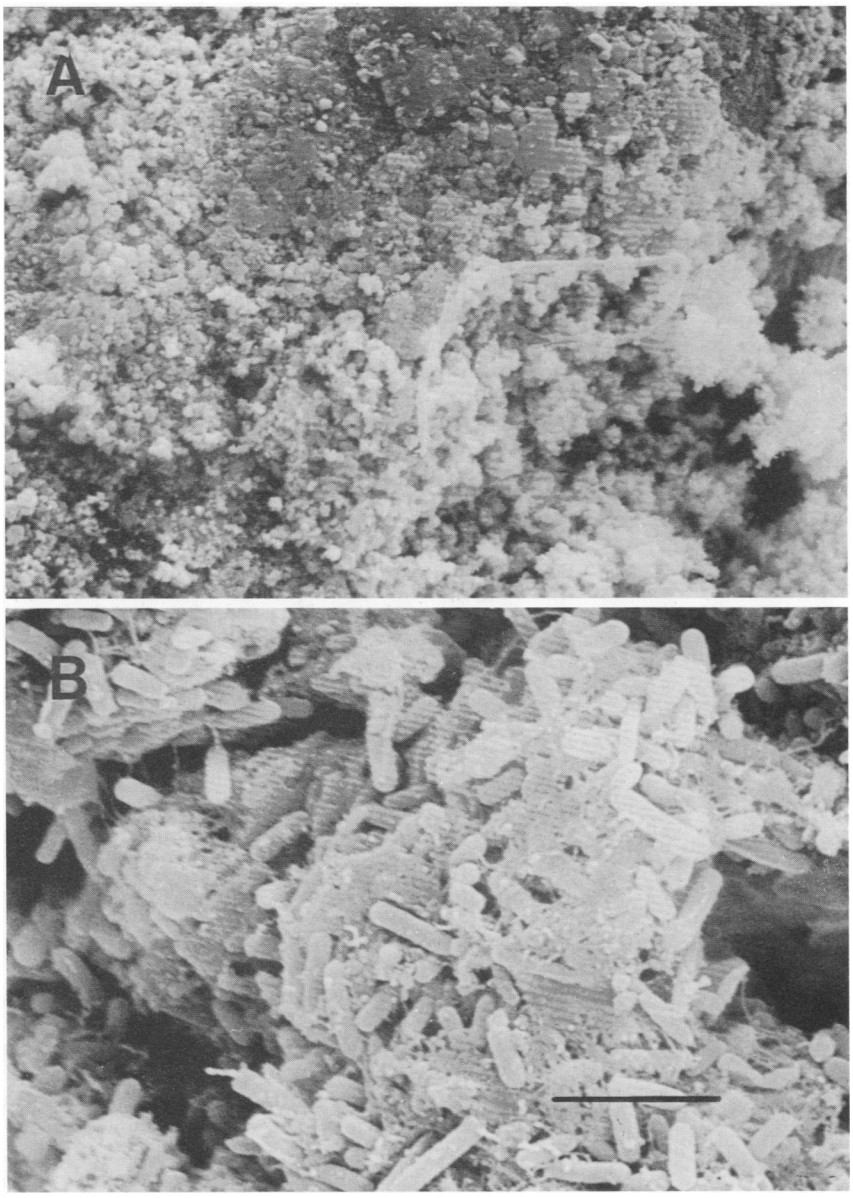

FIG. 5. Scanning electron microscopy of ileal biopsies from control and colonized animals. In contrast to the noncolonized control tissue, in which no bacteria were seen (A), numerous bacteria were present in the mucus layer surrounding the crypts of colonized mucosa (B). Bar = 5 μm.

crase activity are shown in Fig. 4. Paired biopsies were taken from 10 rabbits before inoculation and again 72 h after inoculation for mucosal sucrase activity. Our results showed no consistent or significant change in sucrase activity from the activity in the original biopsy (mean = 35% increase) in the animals that were controls or that were colonized with *E. coli* at $<10^4/cm^2$. In contrast, the five animals that were colonized with *E. coli* at greater than or equal to $10^4/cm^2$ revealed a consistent and significant decrease (mean = 44% decrease) in sucrase activity from the original biopsy in every colonized animal studied ($P < 0.03$ [Student paired t test], $n = 10$ rabbits).

Results of electron microscopy. Scanning electron microscopic studies of the ileal biopsy from an animal colonized with *E. coli* 132-3 (O75ac:H7) (Fig. 5) revealed multiple bacteria in the residual mucus layer surrounding the crypts. In contrast, studies of the ileal biopsy taken from an uncolonized animal (colonized with 1392−) revealed few, if any, bacteria in the mucus layer. Transmission electron microscopy of biopsies from an animal colonized with *E. coli* 132-3 (Fig. 6) showed bacteria in close proximity to intact microvilli, with no evidence of effacement or destruction of the microvilli or evidence of tissue invasion.

DISCUSSION

E. coli strains have long been recognized as potential enteric pathogens. Recently, our concepts of the ways that the versatile *E. coli* can cause diarrhea have been greatly expanded, and most investigators would now agree that

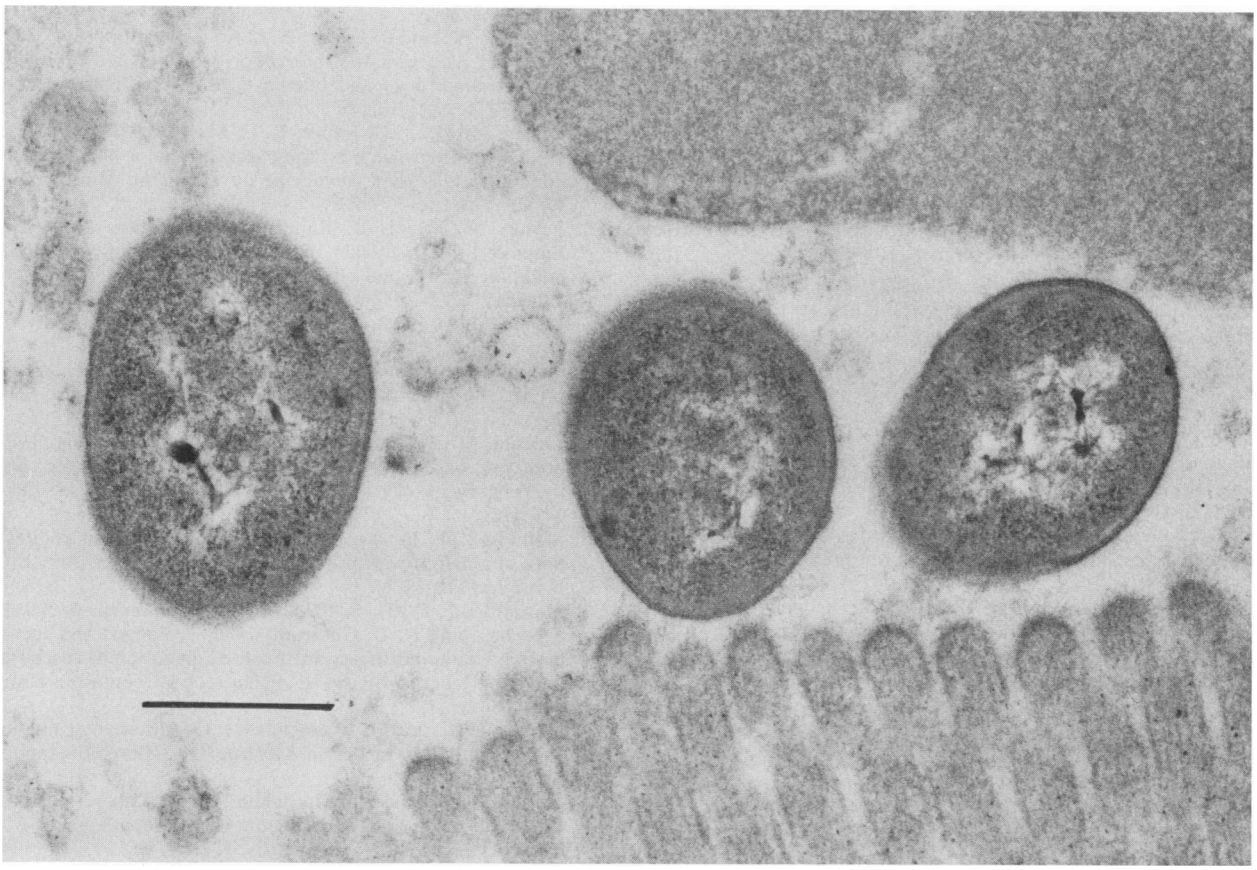

FIG. 6. Transmission electron microscopy of colonized mucosa reveals bacteria in close proximity to intact microvilli, without tissue invasion or brush border effacement. Bar = 0.5 μm.

there are at least six or seven different ways in which *E. coli* can cause diarrhea (28). Represented among the *E. coli* diarrheas is a range of types of infectious diarrheas that include those caused by cholera-like and other enterotoxins (enterotoxigenic *E. coli*), inflammatory colitis caused by invasive organisms (enteroinvasive *E. coli*), diarrheas caused by effacing, enteroadherent organisms (enteropathogenic or enteroadherence factor-positive *E. coli*), and hemorrhagic diarrhea caused by enterohemorrhagic *E. coli* that produce Shiga-like toxin(s).

This study confirms previous findings that nontoxigenic, colonizing *E. coli* cause diarrhea and extends these findings to show that colonizing *E. coli* reverse normal ileal water and electrolyte absorption and reduce mucosal sucrase activity. Our findings of net water and electrolyte secretion 3 days after inoculation do not appear to be due to a secretory enterotoxin produced by the colonizing *E. coli*, as this colonizing *E. coli* (1392^+) failed to elicit any secretion when placed in ligated ileal loops for 6 to 18 h, despite high levels of colonization over these periods (37). While we cannot fully exclude a delayed toxic effect or that of a low level of Shiga-like toxin, currently recognized enterotoxins would have been expected to have had an effect by 18 h in heavily colonized ligated loops. These effects occurred in the absence of tissue invasion and in the absence of any effacement or destruction of brush border architecture such as that seen with attaching and effacing *E. coli*. Our findings of reduced mucosal sucrase activity do not appear to be due to protease degradation of the disaccharidase activity in vitro because the tissues were promptly frozen to −20°C and assayed within 72 h, as was originally described by Dahlqvist (4). It remains possible, however, that well-recognized *E. coli* proteases could, with colonization at the mucosal surface over a 3-day period, alter brush border disaccharidase activity (30). Still other possibilities include alterations of mucosal function by bacterial components (such as colonization factor antigen itself), although these effects were not seen at 18 h in ligated ileal loops (37).

Our findings that colonizing *E. coli* appeared to adhere to the mucus layer and to adhere only relatively loosely to the brush border are consistent with the results of Wanke and Guerrant, showing that these *E. coli* are easily washed from the mucosa (37). This adherence may be similar to that recently described by Yamamoto and Yokota for *V. cholerae* O1, which adheres to the mucus layer of the human small intestine more avidly than to the brush border. Thus, the small intestine mucus layer was a primary adherence target for *V. cholerae* O1 in human infection (38). They speculated, as have other investigators (13, 14, 21, 23, 24), that the small intestine mucus layer may be the site of proliferation of *V. cholerae* O1. They further suggest that "adhesins" of *V. cholerae* O1 are not saturated with "receptors" in the mucus coat, so the organism can move through the mucus layer and attach to the brush border and that gaps exist in the mucus layer that allow the organism to reach the brush border epithelial cells.

Our findings demonstrate that heavy ($\geq 10^4$ CFU/cm^2) colonization of the mucus layer with loose mucosal attach-

ment can impair normal small bowel water and electrolyte transport and alter disaccharidase activity. Whether or not colonization results in clinically overt acute or prolonged diarrhea, these functional alterations in absorption and disaccharidase activity may have important nutritional consequences, especially if they persist over several days. Unlike toxigenic *E. coli*, which typically cause an acute dehydrating illness in the host, nontoxigenic colonizing *E. coli* consistently cause functional derangements as noted but cause acute overt diarrhea in only 10 to 60% of the hosts (15, 17, 37). Potentially important host differences, as well as the specific microbial traits and mechanisms responsible for these functional alterations, require further study.

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