Bacteria and the Mucus Blanket in Experimental Small Bowel Bacterial Overgrowth

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Self-filling blind loops were created experimentally in jejunal segments of specific pathogen-free male Wistar rats, and the loop contents and mucosa were examined over an 8-week period for evaluation of the interaction between mucus and luminal bacteria. Corresponding jejunal segments from rats that did not undergo surgery were used as controls. Proliferation of anaerobic bacteria developed in the test animals by the first week after surgery. Despite anaerobic bacterial proliferation, no adherence by bacteria to the intestinal microvillus surface was observed by scanning or transmission electron microscopy. Rather, bacteria were present within the mucus layer overlying the intestinal mucosal surface. Immunoassay of goblet cell mucin demonstrated an increase in the proportion of mucin present in the intestinal lumen and a decrease in mucin levels in the jejunal mucosa. These results suggest that the interaction of bacteria with mucus is an important mechanism of protection of the mucosal surface in experimental small bowel bacterial overgrowth. (Am J Pathol 1987, 126:527–534)

THE SEQUELAE of the contaminated small bowel syndrome can be induced experimentally in self-filling blind loops (SFBLs) by causing the abnormal proliferation of anaerobic bacteria, of the type normally seen in the colon, in the lumen of the upper small intestine. Patchy changes in the surface membrane structure and mitochondria of epithelial cells,[1–3] decreased disaccharidase-specific activities,[4–6] and impaired monosaccharide transport[4,7] develop within the jejunum of SFBLs. However, a number of studies have failed to provide evidence of invasion by bacteria[1–3,8] or the production of classic bacterial enterotoxins.[9] Adherence of bacteria to the microvillus membrane surface of enterocytes in the SFBLs, which appears to be a prerequisite for the initiation of the effects of recognized enteric pathogens,[10] has not been found. The absence of microvillus membrane colonization suggests that host factors might prevent bacterial contact with the membrane. The overlying mucus gel could fulfill this function by acting as a viscous physical barrier.[11]

We undertook the present studies to examine the relationship of bacteria proliferating in the jejunum of SFBL and the mucus layer overlying the mucosal surface.

Materials and Methods

Animals

Specific pathogen-free male Wistar rats (Woodlyn, Guelph, Ontario) weighing 150–200 g at the start of the experiments were housed individually in cages with wire mesh floors.

Experimental Procedure

With the animals under enflurane (Ethrane, Ohio Medical Co., Toronto, Ontario) anesthesia, self-filling blind loops 8 cm in length were created surgically 7 cm distal to the ligament of Treitz, as described by Cameron et al.[12] Corresponding proximal jejunum from rats that did not undergo surgery was used as

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control tissue. One to 8 weeks after surgery rats were fasted overnight and killed by cervical dislocation under light ether anesthesia. The abdomen was opened, and the blind loop or a corresponding 8-cm segment of jejunum from control animals was closed at each end with sterile clamps and removed. Eight control rats and 8 rats with blind loops were studied at each of 1, 2, 3, 4, 6, and 8 weeks after surgery. Blind loop and control jejunal segments were flushed with 10.0 ml sterile phosphate-buffered saline (PBS), pH 7.4 at 37 C. Each segment was then opened longitudinally, and blotted, and the mucosa was removed by light scraping with a microscope slide. The scrapings were weighed, placed in 100 vol of 5 mM Na EDTA, brought to pH 7.4 with 0.1 N NaOH, and homogenized for 25 seconds in a Waring blender. All operations were conducted at 4 C. Sodium azide (0.02% final concentration), phenylmethylsulfonylfluoride (PMSF, 0.5 mM), and aprotinin (Trasylol, Sigma Chemical Co., St. Louis, Mo, 500 kIU/ml, final concentration) were added to lumen and homogenate samples before storage. Samples were stored at −70 C.

Bacterial Counts

Luminal contents were serially diluted tenfold in oxygen-free, sterile thioglycollate broth. Dilutions were streaked onto prereduced sheep blood agar plates and incubated anaerobically at 37 C for 72 hours in a Gas Pak anaerobic jar (BBL Microbiology Systems, Mississauga, Ontario).[6,13] The numbers of strict and facultative anaerobic organisms present in the intestinal lumen ($\log_{10}$ of colony-forming units [CFUs] per milliliter of washings) were calculated from the number of CFUs present on blood agar plates. Representative colonies were subcultured onto blood agar plates and incubated for 24 hours at 37 C under both aerobic and anaerobic atmospheric conditions for determining the number of facultative and strict anaerobic bacteria. Bacteria growing only in an anaerobic setting were counted as strict anaerobic bacteria, and those capable of growing aerobically were counted as facultative anaerobes.

Microscopy

Samples for both light and electron microscopy were processed with and without washing with PBS, pH 7.4 at 37 C. For light microscopy, 1-cm segments were excised and fixed overnight in 10% buffered formalin, and 5-μ sections were stained with periodic acid–Schiff (PAS) reagent. In the lower two-thirds of villi chosen for their correct orientation to crypts, goblet cells were counted as the number per 100 columnar cell nuclei.

For electron microscopy, intestinal segments were fixed overnight in 2.5% glutaraldehyde or Karnovsky's fixative in 0.5 M cacodylate buffer at 4 C,[14] postfixed in 1% osmium tetroxide, 0.1 M cacodylate buffer for 2 hours at 4 C, and then washed three times in cacodylate buffer. Samples for scanning electron microscopy were dehydrated in ethanol, critical point-dried, mounted on stubs, sputter-coated with gold, and examined in a JEOL 35 scanning electron microscope at an accelerating voltage of 20 kv. For transmission electron microscopy specimens were dehydrated in ethanol and embedded in Epon, and sections were stained with uranyl acetate and lead citrate. Specimens were examined in a Philips 300 transmission electron microscope at 60 kv. Specimens embedded in Epon were also prepared for light microscopy by removing the Epon with saturated Na ethoxide, and 1-μ sections were stained with PAS.

Assays

Protein was measured by the method of Lowry et al[15] with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo) as standard. Mucin was measured by immunoassay as described previously[16] in stored samples containing protease inhibitors for minimizing mucin degradation.

Statistics

Results for each experimental group are expressed as the mean ± standard error of the mean (SE). Statistical analysis of the differences between means was determined by the two-tailed, nonpaired Student t-test.[17]

Results

The number of anaerobic bacteria in the lumen of the self-filling blind loop was markedly increased when compared with that in normal jejunum as early as the first week after surgery. The total number of strict anaerobic bacteria, expressed as $\log_{10}$ of CFUs per milliliter, increased from 4.40 ± 0.41 in normal jejunum to 9.17 ± 0.35 within the blind loop 1 week after surgery ($P < 0.001$). At the same time, total facultative anaerobic bacteria increased from 6.07 ± 0.24 to 8.47 ± 0.24 $\log_{10}$ CFU/ml ($P < 0.01$). Thereafter, the number of anaerobic bacteria in the blind loops remained relatively stable at 10^8–10^{10} CFU/ml (Table 1).[6]

Scanning electron microscopy of normal rat jejunum, fixed *in situ* without washing, revealed an incomplete and very thin mucus coat overlying the villus surface (Figure 1). Leaflike ridges of villi, nor-

Table 1—Total Numbers of Strict and Facultative Anaerobic Bacteria Present in Washes of Control and Blind Loop Jejunum*

	Strict anaerobes	Facultative anaerobes
Control Jejunum	4.40 ± 0.41	6.07 ± 0.24
Blind loop jejunum (weeks after surgery)		
1	9.17 ± 0.35	8.47 ± 0.24
2	9.58 ± 0.34	9.36 ± 0.18
3	8.33 ± 0.41	9.27 ± 0.14
4	9.74 ± 0.48	11.35 ± 0.18
6	10.72 ± 0.22	9.51 ± 0.51
8	9.48 ± 0.32	9.94 ± 0.40

*Log_{10} CFU/ml, mean ± SE. All blind loop results are significantly increased from levels in the control groups ($P < 0.01$).

mally found in the rat jejunum,[18] were clearly seen under the mucus layer and in areas free of mucus. The mucus layer was easily removed when the unfixed intestine was washed with saline. Rats are coprophagous, and the upper small bowel normally contains relatively larger numbers of bacteria (10^4–10^6). However, most bacteria were found in clumps entangled within threads of mucus (Figure 2) and were rarely seen attached to the underlying microvillus surface.

In marked contrast to the normal rat mucosa, scanning electron microscopy of the blind loop jejunum revealed a thick, confluent layer of mucus that obscured the underlying villus architecture in an almost continual blanket (Figure 3). Large numbers of organisms were present in the thick mucus blanket (Figure 4). As in normal rat jejunum, bacteria appeared to be entangled in threads of mucus, rather than attached directly to the underlying microvillus surface. These findings were noted as early as the first week after surgery, and no temporal changes were noted by electron microscopy.

More precise localization of bacteria with respect to the microvilli and mucus layer was achieved by transmission electron microscopy. Microorganisms were exclusively present in mucus exterior to the glycocalyx microvillus membranes (Figure 5). Bacteria in the mucus layer were not aligned along the interface with the fuzzy coat, but were evenly distributed throughout. The identity of the mucus layer is attested to by its location superficial to the fuzzy coat, its continuity with the discharging contents of intestinal goblet cells (not shown), and the fact that it stained positively with PAS (not shown). Study of 150 photomicrographs from representative areas of blind loop jejunum revealed no case of close apposition of microorganisms

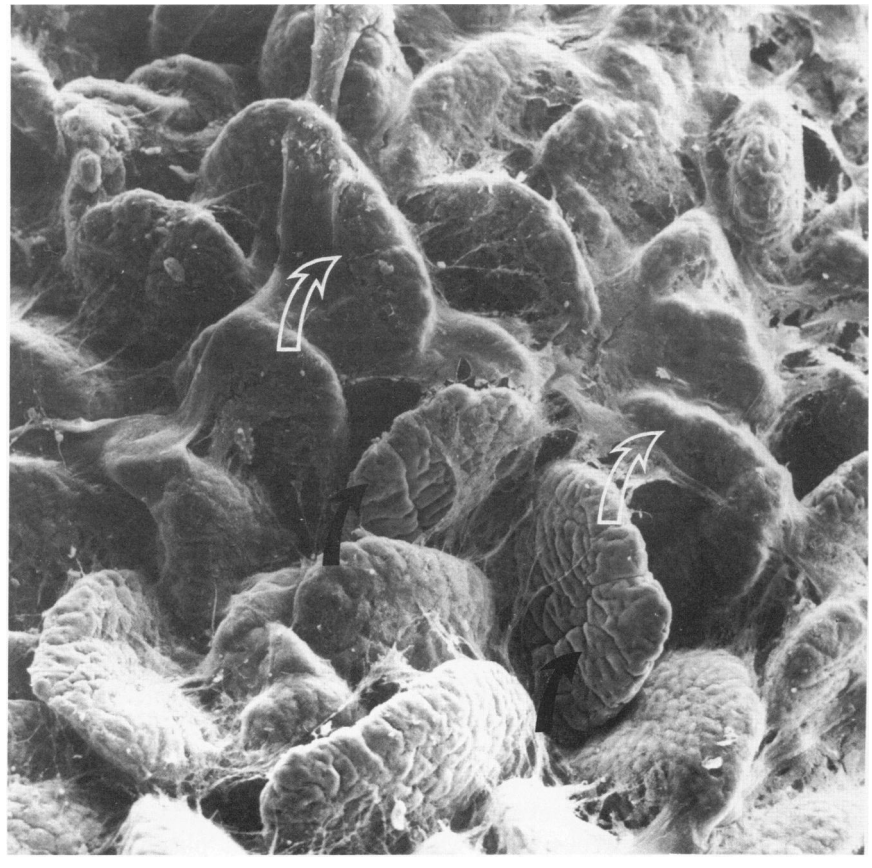

Figure 1—Scanning electron micrograph of control rat jejunum. A thin mucus layer overlies ridgelike villi (*open arrows*). Villi are seen clearly in areas where the overlying mucus has been disrupted (*solid arrows*). (×100)

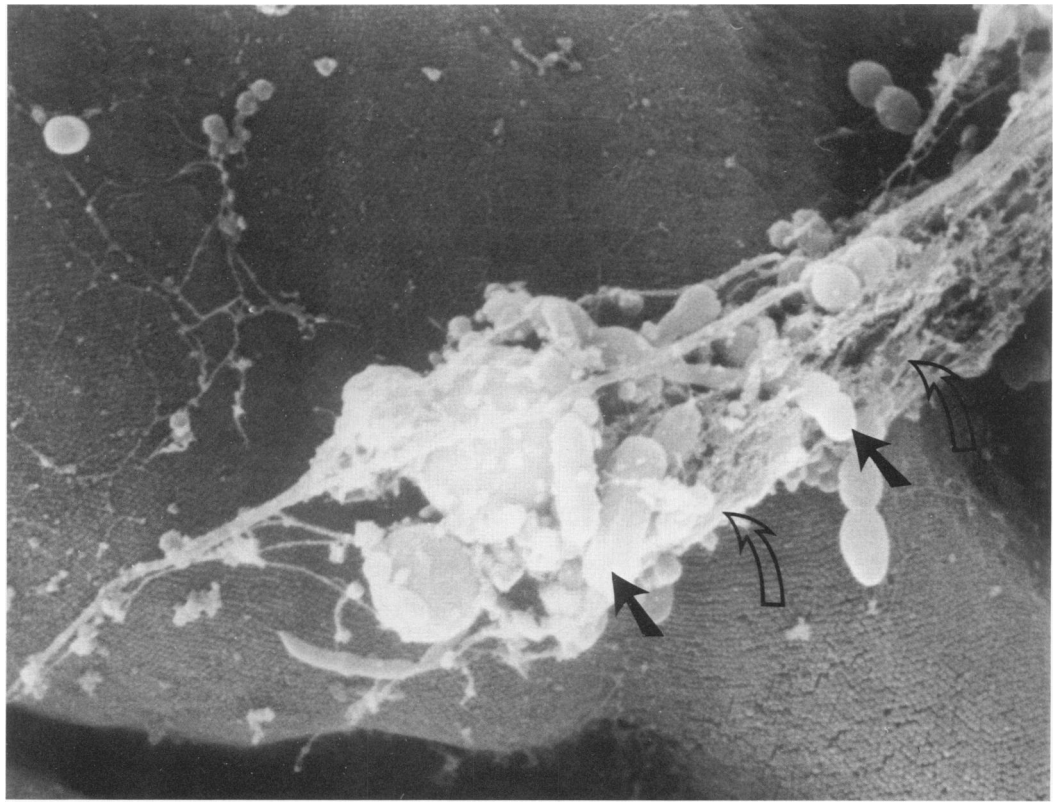

Figure 2—Scanning electron micrograph of control rat jejunum. Pleomorphic bacteria (*solid arrows*) are entangled in threads of mucus (*open arrows*). No bacteria are adherent to the exposed underlying microvillus surface. (×14,000)

to the intestinal enterocyte membrane. Changes such as microvillus disruption and effacement or "cupping" of the microvillus plasma membrane, commonly seen in infections due to enteroadherent organisms,[19,20] were not observed. Thus, although large numbers of bacteria were randomly distributed throughout a greatly thickened mucus layer, bacteria did not penetrate beyond this barrier to adhere to or colonize the microvillus surface.

The number of PAS-positive goblet cells per 100 columnar cell nuclei was not significantly changed in blind loop intestine 4 weeks after surgery (13.8 ± 1.3 in blind loop versus 12.0 ± 0.7 goblet cells in normal rat intestine, $P > 0.05$).

The total amount of immunoassayable mucin in the mucosal homogenate and luminal washings was not statistically different from control levels at 2 weeks after blind loop formation (3.4 ± 0.34 mg/8-cm blind loop segment, 4.23 ± 0.62 mg/8-cm control segment, $P > 0.05$). At 4 and 8 weeks after surgery, however, total mucin was significantly reduced in blind loop segments (4 weeks, 2.03 ± 0.50 mg, $P < 0.05$; 8 weeks, 1.93 ± 0.53 mg, $P < 0.05$).

As shown in Figure 6A, even though total mucin was unchanged at 2 weeks, there was a marked increase in luminal mucin ($P < 0.01$) that persisted at 4 and 8 weeks. As a percentage of the total mucin, luminal mucin increased from 2.4% ± 0.6% in control jejunum to 18.5% ± 3.9% in blind loops 2 weeks after surgery ($P < 0.001$), 20.5% ± 4.5% at 4 weeks ($P < 0.001$), and 28.0% ± 6.8% ($P < 0.001$) 8 weeks after surgery.

In contrast, mucosal mucin fell progressively and significantly with the age of the blind loop (Figure 6B), accounting for all of the decrease in total mucin.

Discussion

Although the presence of an increased number of anaerobic bacteria is a well-recognized consequence of experimental blind loop formation,[4,6] the luminal localization of anaerobic bacteria has not previously been characterized. Our electron microscopy results indicate that bacteria in the lumen of self-filling blind loops are present within the mucus layer overlying the intestinal mucosal surface. Microorganisms were distributed evenly and apparently randomly throughout the layer without evidence of congregation at the mucosal interface. In addition, effacement of mucosal plasma membranes, which has recently been de-

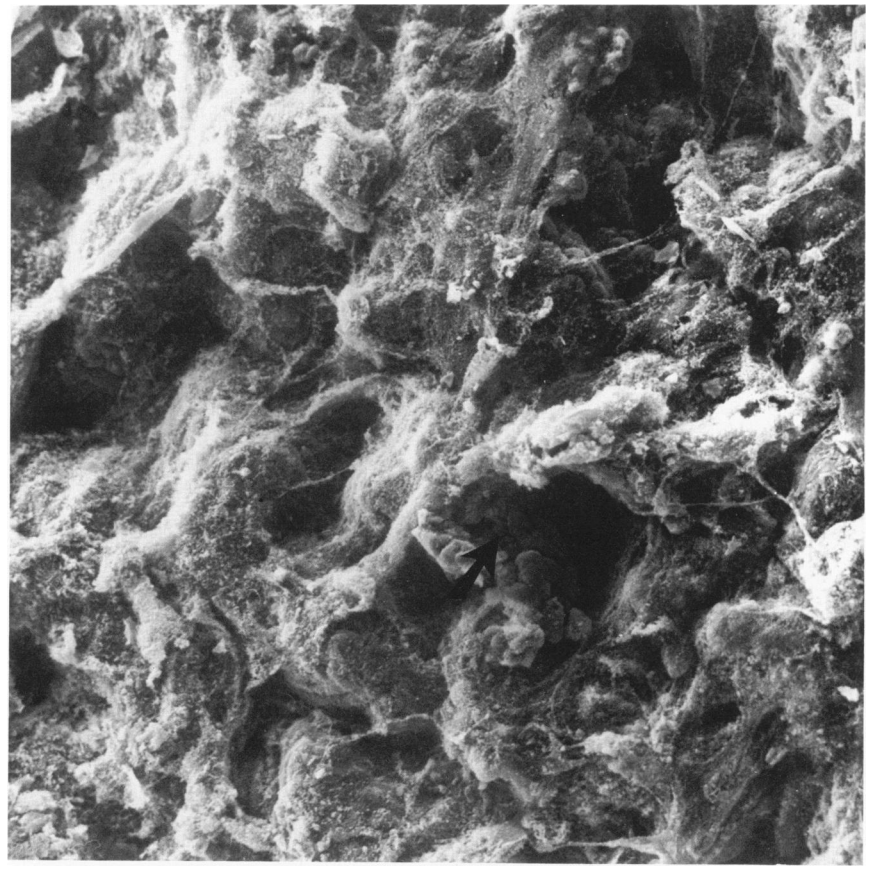

Figure 3—Scanning electron micrograph of rat blind loop jejunum 4 weeks after surgery. A markedly increased mucus layer overlies the intestine, compared with that in control jejunum (shown in Figure 1). The underlying villi are exposed only in a few areas, such as indicated by the *solid arrow*. (×125)

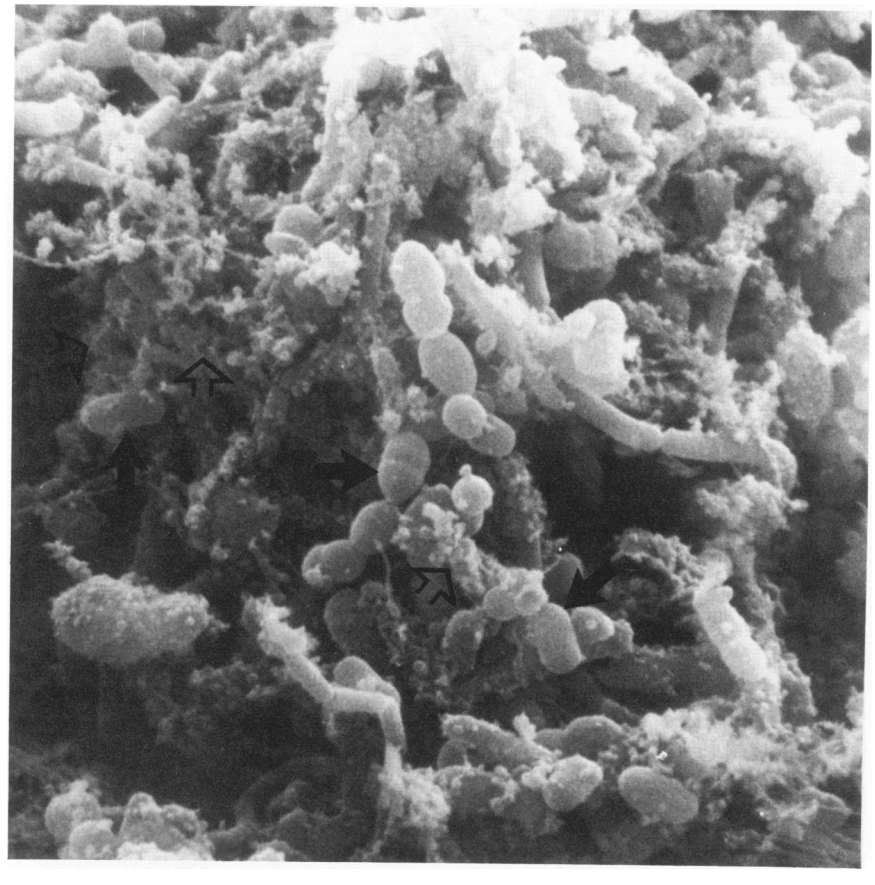

Figure 4—Scanning electron micrograph of rat blind loop jejunum 4 weeks after surgery. Increased magnification of intestine shown in Figure 3 demonstrates large numbers of microorganisms (*solid arrows*) within the mucus layer (*open arrows*). (×11,100)

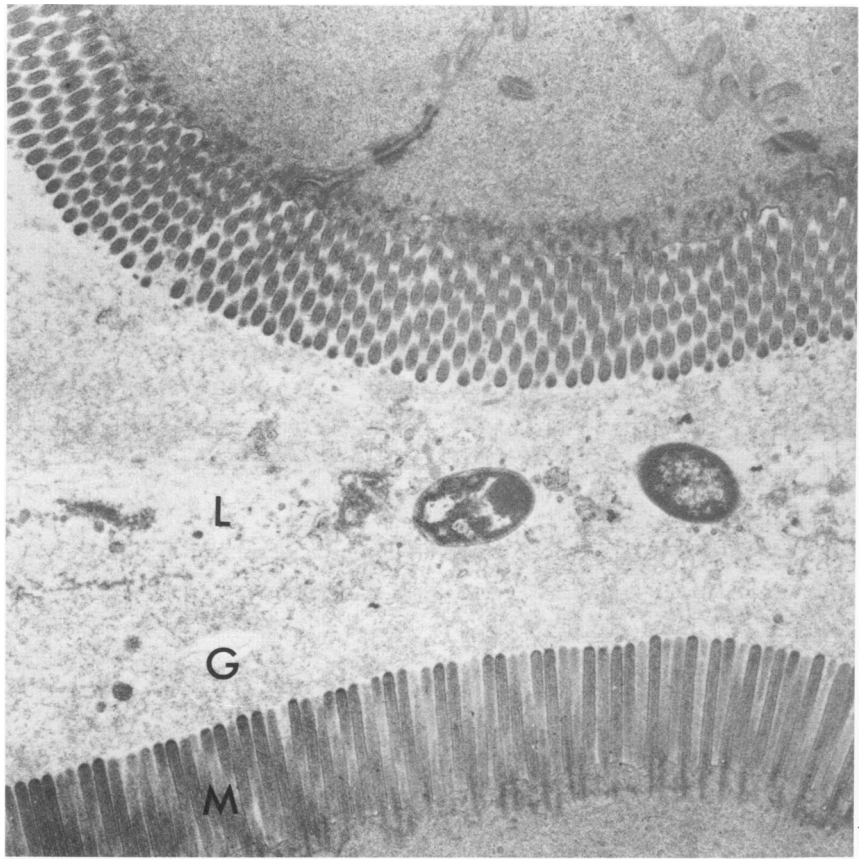

Figure 5—Transmission electron micrograph of rat blind loop jejunum 4 weeks after surgery. Bacteria are present in the intestinal lumen (*L*) superficial to the surface glycocalyx (*G*) and microvilli (*M*) of mucosal enterocytes. (×14,000)

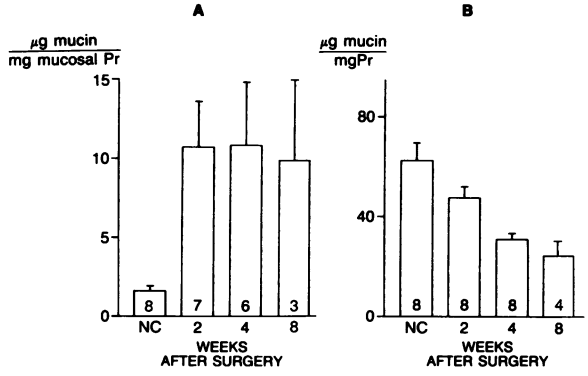

Figure 6—Immunoreactive mucin per mucosal protein (micrograms per milligram) present in the lumen of control jejunum (*NC*) and blind loop jejunum 2, 4, and 8 weeks after surgery (mean ± SE). Levels at 2, 4, and 8 weeks were significantly increased, compared with those of control segments, $P < 0.01$. Numbers in each histogram indicate the number of samples within each group used for data analysis. **B**—Immunoreactive mucin per mucosal protein present in the jejunal mucosa of control rats (*NC*) and rats with blind loops 2, 4, and 8 weeks after surgery. Levels of mucin are decreased 2 weeks ($P < 0.05$), 4 weeks ($P < 0.01$), and 8 weeks ($P < 0.01$) after surgery, compared with levels in control segments.

scribed during enteric infection by specific enteroadherent pathogens such as enteropathogenic *Escherichia coli*,[20] was not observed. We therefore found no evidence of selective binding of bacteria to the intestinal mucosa. Absence of enteroadherent organisms has been reported in five children with small bowel bacterial contamination.[20] Several studies in experimental self-filling blind loops have also remarked on the absence of bacterial enteroadherence.[2,3,8] Hartley also found that microorganisms in the terminal ileum and colon were located in the mucus layer, rather than directly adherent to the mucosal surface,[21] which suggests that commensal intestinal flora are normally attracted to, or restrained by, luminal mucus. Because our results relate to anaerobic bacteria, which are normal constituents of the bacterial population of the colon, it would appear that these bacteria select the mucus habitat when colonizing either the colon or small intestine.

Accumulation of microorganisms within mucus probably depends upon several factors. Anaerobic bacteria produce glycosidases,[22,23] which are capable of cleaving carbohydrate residues present in high-molecular-weight mucus glycoprotein or mucins. Released carbohydrate may attract bacteria as a nutritional substrate. Similarly, proteases produced by bacteria may degrade peptides contained within the mucus gel and provide a nitrogen source suitable for bacterial utilization. In addition, there is considerable evidence that anaerobic bacteria may bind through bacterial adhesins to specific receptors present within the complex mucus layer. Cohen demonstrated the ability of both commensal organisms[24] and enteric pathogens[25] to bind to luminal mucus obtained from the colon of mice. Williams and Gibbons[26] have shown that salivary mucus inhibits attachment of endogenous bacteria in the oral cavity to buccal epithelial cells by competitive binding.

Our results indicate that shortly after creation of experimental self-filling blind loops there is a significant increase in luminal mucin and a corresponding fall in mucosal mucin. These findings are consistent with stimulation of goblet cell mucin secretion and suggest that the thickened mucus layer does not arise simply by accumulation within the blind loop. Indeed, it is possible that commensal bacteria actually stimulate mucin secretion. Previous studies have shown that mucin secretion can be stimulated by specific enteric pathogens[22] and by bacterial enterotoxins.[27]

The observation that bacteria are evenly distributed throughout the depth of the mucus layer suggests that mucus acts not as a permeability barrier for bacteria, but as a retention zone which restricts their free access to the underlying mucosa. Although mucus appears to prevent attachment of bacteria to the mucosal surface, injury to the underlying villus absorptive cells in the experimental blind loop is nevertheless observed. Morphologic evidence of patchy structural changes in both enterocyte brush borders and cytosol constituents has been described both in man[1] and in experimental self-filling blind loops.[2,3] In addition, loss of brush border disaccharidase activities[28] and impaired glucose transport[4] have been reported as a consequence of anaerobic bacterial proliferation in the SFBLs. Thus, interactions of mucus and bacteria do not completely preserve mucosal enterocyte structure and function. Anaerobic bacteria present in small bowel bacterial overgrowth elaborate proteases that are capable of either releasing[29] or destroying[30] brush border disaccharidase activity. Such proteases may be released from anaerobic bacteria that colonize luminal mucus and then permeate the mucus layer before acting on ectoenzymes present on the surface of enterocyte microvillus membranes.

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