Role of intestinal mucus in transepithelial passage of bacteria across the intact ileum in vitro

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Background. *Although gastrointestinal mucus is one of a number of putative host defense mechanisms that protect the gut barrier against microbial translocation, little experimental data are available to show its role in this process. The present study sought to determine the role of mucus depletion on the transepithelial passage of bacteria across viable segments of rat ileum mounted in Ussing chambers in vitro.*

Methods. *Intestinal mucus was depleted in 12 rats after injection with pilocarpine (100 mg/kg intraperitoneally) 45 minutes before intestinal harvest. The mucosal surfaces of the perfused gut segments mounted in the Ussing chamber were exposed to 5×10^9 CFU/ml Escherichia coli (C-25). Viability was monitored by continuous measurements of the potential difference generated by the membranes. The electrical characteristics were unaltered by pilocarpine pretreatment or exposure to bacteria.*

Results. *Bacterial passage occurred in 100% of pilocarpine membranes as compared with 33.3% of controls ($p < 0.05$). Pilocarpine-treated membranes resulted in 19.9 ± 7.5 mg of retrievable mucus as compared with 28.8 ± 7.2 mg in controls ($p < 0.05$). Light and transmission electron microscopy revealed an intact epithelial surface in all membranes. There was a marked decrease in mucus on the surface of pilocarpine-treated membranes.*

Conclusions. *Intestinal mucus secretion is a critical factor in the barrier function of the gut, and its depletion results in a dramatic increase in bacterial passage across the intact rat ileum. (SURGERY 1994;116:76-82.)*

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THERE IS INCREASING EVIDENCE THAT intestinal barrier failure may play a role in the development of systemic infection in victims of immunosuppression, burns, and trauma through a process known as bacterial translocation.[1, 2] This phenomenon may result from local or systemic insults that may induce an alteration of one or more of the components of the gut barrier (e.g., mucus layer, glycocalyx, immunoglobulins, tight junctions, proteolytic enzymes, intestinal peristalsis, epithelial desquamation). Gastrointestinal mucus is one of several putative host defense mechanisms that comprise the gut barrier.[3] Mucus is postulated to form a physical barrier against the translocation of bacteria between the intestinal lumen mainly by preventing bacterial adhesion to the epithelial cells, a prerequisite for internalization or invasion.[4] Alternatively, it may function as a chemical trap by providing specific binding sites for bacterial adhesions.[5] If intestinal mucus mediates bacterial attachment, then depletion of intracellular mucus should increase bacterial interaction with epithelial cell surfaces and thus increase bacterial translocation. The present study was designed to determine the effect of mucus depletion on bacterial translocation across the ileal mucosa by using the Ussing chamber.[6] Among the various stimulants to mucus secretion, cholinergic agents have been shown to deplete goblet cell mucus by increasing exocytosis.[7] Specian and Neutra[8] showed that intraperitoneal administration of the parasympathomimetic agent pilocarpine will deplete rat intestinal goblet cells of 50% to 100% of their mucus depending on the dose, the intestinal segment studied, and the time

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between its administration and specimen procurement. We therefore used pilocarpine to promote mucus depletion in vivo before preparing ileal mounts for translocation studies with *Escherichia coli*.

MATERIAL AND METHODS

Ussing system. The Ussing system has been described in detail elsewhere.[6, 9] In this system the muscle layers are stripped from fresh intestinal segments, and the mucosa-submucosa is suspended between two oxygenated perfusion chambers. Isolated perfusion of the mucosal and submucosal surfaces is accomplished through an oxygenated perfusate of Dulbecco's modified Eagle's medium (Sigma Chemical Company, St. Louis, Mo.) containing 20 mmol/L L-glutamine (Sigma Chemical Company). The reservoirs are jacketed with a recirculating water bath that maintains the perfusate temperature at 37° C. The viability of the membrane can be monitored during an experiment of several hours by continuous recording of the potential difference across the membrane[10] with two calomel AgCl electrodes connected to a voltmeter (DVC 1000; World Precision Instruments, New Haven, Conn.). Mucus secretion by intestinal epithelial cells is maintained in this system. Our preliminary experiments showed retrieval of an average of 29.1 ± 7.5 mg of mucus per intestinal membrane after 3 hours of perfusion. All components of the Ussing system are sterilized before experimentation, allowing quantification of bacterial passage from the mucosal to submucosal reservoir after inoculation of bacteria. Sterility is verified by culturing the perfusate before the start of each experiment.

Experimental protocol. Membranes were prepared from 12 rats injected with pilocarpine (160 mg/kg intraperitoneally) 45 minutes before intestinal harvest or with an equal volume of saline solution (controls). The dose of pilocarpine had been shown to deplete intestinal goblet cell mucus within 30 to 45 minutes.[8] Anesthesia was induced with inhaled ether and maintained with pentobarbital (40 mg/kg intraperitoneally). A midline laparotomy was performed, and the distal 10 cm of terminal ileum free of Peyer's patches was resected. The excised bowel was opened along the antimesenteric border and rinsed in Hank's balanced salt solution (HBSS; GIBCO, Grand Island, N.Y.) to remove the intraluminal contents. The seromuscular layer was dissected from the submucosa-mucosa layers with the assistance of a dissecting microscope and discarded. The prepared tissue was mounted in the Ussing chamber. After a 15-minute period of stabilization and perfusion, *E. coli* (5×10^9 CFU/ml) was added to the mucosal reservoir. Serial cultures of the submucosal reservoir fluid were taken at 60-, 120-, and 180-minute intervals to check for bacterial passage. We have found that bacteria will pass predictably across the rat distal ileum from the mucosal to the submucosal chamber in approximately one third of the normal membranes during a 3-hour period.[9, 11] The potential difference generated by the tissue, a measure of viability, was continuously monitored throughout the course of every experiment. Ileal mucosal mucus was measured at the end of each experiment. Random membranes from both groups were examined by scanning and transmission electron microscopy.

Animals. Specific pathogen-free male rats (Harlan Sprague-Dawley, Indianapolis, Ind.) weighing 250 to 350 gm were housed in plastic cages with pine bedding and fed commercial rat chow (Purina Mills Inc., St. Louis, Mo.) and tap water at pleasure for a maximum of 5 days before experimentation. All procedures were approved by the Children's Hospital of Pittsburgh Animal Care Committee.

Preparation of bacteria. *E. coli* C-25 was provided by Drs. Edwin Deitch and Rodney Berg (Shreveport, La). This strain is streptomycin and bacitracin resistant and is known to translocate in vivo without causing overt disease.[12] It causes no detectable damage in the Ussing chamber.[9] The bacteria were grown in trypticase soy broth at 37° C for at least 20 hours before experimentation. On the day of experimentation the bacterial suspension was centrifuged, and the pellet was washed twice in HBSS. The final pellet was suspended in HBSS to a final concentration of 5×10^9 CFU/ml.

Determination of bacterial passage. Immediately after inoculation with *E. coli*, 0.5 ml samples were taken from the mucosal and submucosal reservoirs. Serial submucosal reservoir samples were taken after 60, 120, and 180 minutes. A second mucosal reservoir sample was taken at 180 minutes. The residual fluid in the submucosal reservoir was centrifuged and 0.5 ml was submitted for quantitative culture; this was referred to as the 180 total sample. Serial sample dilutions were made, and two 100 μl aliquots were plated on MacConkey agar. To ensure identification of *E. coli* C-25, additional samples were plated on lactose-free MacConkey agar containing 100 mg/ml streptomycin sulfate. All plates were incubated aerobically at 37° C for 48 hours. Results were expressed as colony-forming units per milliliter of fluid (CFU/ml). The 180 total sample was expressed as colony-forming units per milliliter of the total amount of residual fluid. Bacterial passage was said to have occurred when the submucosal reservoir cultures at either 60, 120, or 180 minutes were positive or when the 180 total sample yielded more than 10 CFU/ml in the face of prior negative cultures.

Exclusion criteria. Exclusion criteria for the ileal mounts were established based on extensive prior preliminary experiments. Ischemic time, the time between clamping the mesenteric vasculature and membrane perfusion in the Ussing system, must be 10 minutes or

Table I. Incidence of bacterial passage and amount of mucus retrieved from pilocarpine-treated and control animals

	Incidence of bacterial passage	*Mucus (mg)**
Pilocarpine	12/12 (100%)†	19.9 ± 7.5‡
Controls	4/12 (33.3%)	28.8 ± 7.2

*Values are mean ± SD.
†$p < 0.05$ compared with controls
‡$p < 0.05$ compared with controls.

less to obtain reproducible data. The initial potential difference should be greater than 3.0 mV to ensure a viable membrane at the conclusion of the experiment. All cultures for bacterial passage were only valid if the initial perfusate (before bacterial inoculation) and the submucosal reservoir immediately after inoculation were sterile. The experiment was terminated prematurely if the submucosal reservoir became cloudy at any time. This was indicative of either a leak or a hole in the membrane.

Quantification of mucus. After 3 hours of perfusion in the presence of bacteria, the Ussing chamber was opened and the mucosal surface of the membrane was exposed. The mucus on the surface of the epithelial layer of the rat ileal segment was retrieved, aided by the use of the dissecting microscope. The weight of the collected mucus (milligrams) from each of 10 membranes in control and experimental groups was determined. Mucus was not collected from two sets of membranes submitted for histologic examination.

Histologic findings. Membranes were randomly selected and fixed in situ in the Ussing chamber. All fixations were carried out with 2.5% glutaraldehyde in phosphate-buffered saline solution for 1 hour at 4° C. After fixation, membranes were washed three times in Sabatini's solution (phosphate-buffered saline solution with 6.8% sucrose) and cut into small (1 mm^3) cubes. All samples were then postfixed with 1% osmium tetroxide for an additional hour followed by three washes in Sabatini's solution. The samples were passed through a graded series of alcohols (30%, 50%, 75%, 90%, 100%; 15 minutes each) followed by treatment with propylene oxide (15 minutes) for pellets, a 1:1 epon–propylene oxide mix (1 hour), and three changes in pure epon (3 hours, 3 hours, and overnight). Samples were oriented in a longitudinal plane and embedded in Ladd (Ladd Research Industries, Burlington, Vt.) embedding trays. Polymerization occurred at 64° C overnight. Serial ultrathin (60 nm) sections of selected areas were cut, mounted on grids, and double stained with 2% uranyl acetate (7 minutes) and 1% lead citrate (3 minutes). Visualization was carried out with a JEOL 100CX (JEOL USA, Inc., Peabody, Mass.). All membranes were examined by a single investigator who was blinded to the groups from which the membranes were derived.

Data analysis. All quantitative bacterial culture results were log transformed before analysis and expressed as $\log_{10}$ colony-forming units per milliliter. Statistical analyses were performed with Statgraphics Version 5.0 software (STSC Inc., Rockville, Md.). The potential difference, the measured mucus, and the quantitative bacterial passage are expressed as the mean ± standard deviation for each group of ileal membranes. The Student two-tailed *t* test was used to evaluate differences in means between groups. The incidence of bacterial passage was compared between groups with chi-squared analysis. Probabilities less than 0.05 were considered significant.

RESULTS

The administration of pilocarpine resulted in a consistent visible discharge of mucus from the eyes, mouth, and intestine (as evidenced by prompt diarrhea in all animals. No rat died after the administration of pilocarpine. The amount of mucus collected from the mucosal aspect of the membrane at the conclusion of the experiment was significantly lower in the pilocarpine group than in the controls ($p < 0.05$; Table I). Membranes from pilocarpine-treated animals exhibited a significantly increased incidence of bacterial passage as compared with controls ($p < 0.05$). Quantitative mucosal reservoir cultures showed that the bacterial concentrations were stable and equivalent for both groups at the beginning and the end of each experiment (Table II). The bacterial concentrations in the submucosal reservoir of membranes exhibiting bacterial passage are also shown in Table II (membranes preventing passage are not included). No significant difference was noted in the number of bacteria that passed between the pilocarpine and control groups except at 1 hour.

Both sets of membranes were equally viable as shown by their potential difference curves displayed in Fig. 1. The potential difference gradually fell in all membranes at a rate characteristic of these preparations.[18] There was no significant difference between these groups at any time point.

Scanning electron micrograph of the mucosal face of control membranes revealed that normal surface structure was maintained after 180 minutes of perfusion in the Ussing chamber (Fig. 2, *A*). The villi were leaf-shaped, smooth structures with a cobblestone pattern created by the individual epithelial cells. Abundant strands and veils of mucus were visible between the crypts. Membranes treated with pilocarpine also exhibited normal surface structures, but mucus was completely absent (Fig. 2, *C*). When examined at higher magnification (Fig. 2, *D*), bacillary microbes and filamentous, segmented organisms were evident on the cell

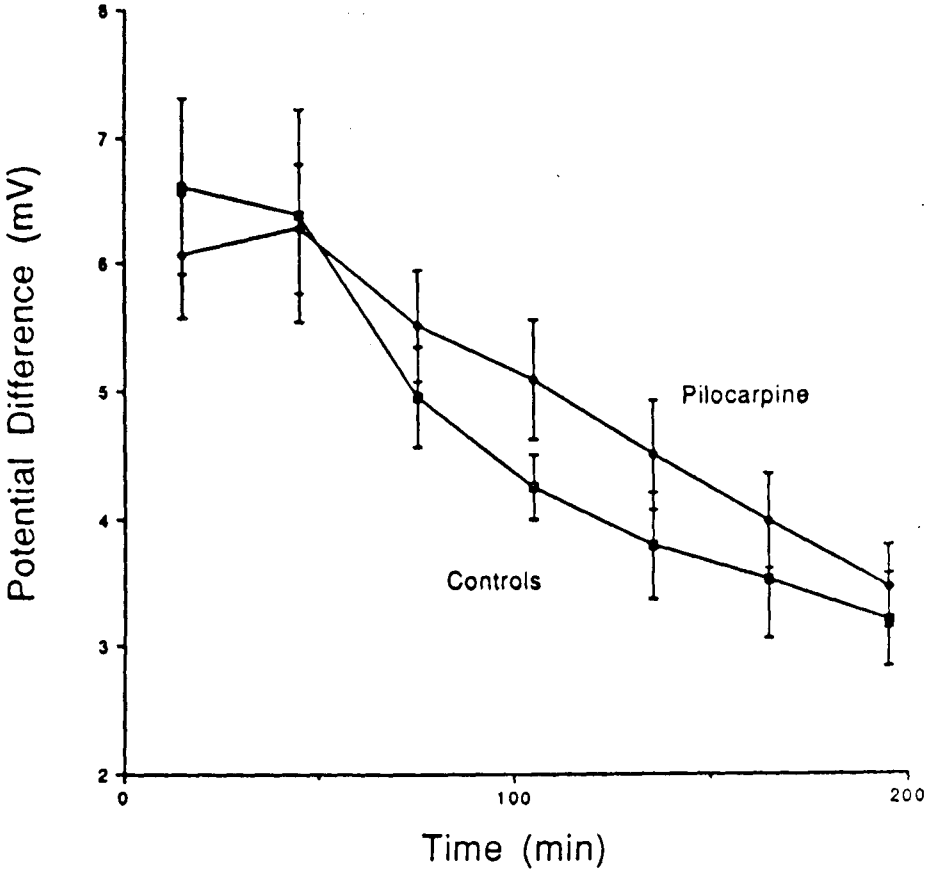

Fig. 1. Potential difference of control versus pilocarpine-treated membranes from time of inoculation with *E. coli* (15 minutes) to conclusion of experiment (195 minutes). Values are mean ± SEM. Potential difference falls at a rate characteristic of this preparation.[9, 11] No significant difference was noted between groups at any time point.

Table II. Mucosal and submucosal reservoir concentrations of *E. coli* in pilocarpine-treated and control animals

	Mucosal reservoir concentration (log_{10} CFU/ml)		*Submucosal reservoir concentration (log_{10} CFU/ml*		
Time (min)	0	180	60	120	180
Pilocarpine	8.64 ± 0.26	8.97 ± 0.24	1.56 ± 0.22	2.61 ± 0.85	3.24 ± 0.7
Control*	8.62 ± 0.17	8.92 ± 0.15	—	3.00 ± 0.56	3.31 ± 0.4

Values are mean ± SD.
*Membranes totally preventing passage of bacteria (8 of 12) were not included in this table.

surface. The filamentous organisms belong to the group of bacteria *Arthromitaceae*, are indigenous to the rat and mouse ileum, and have been described elsewhere[13]; their presence has been confirmed in all of our ileal preparations.

Control membranes examined by transmission electron microscopy appeared normal (data not shown). Membranes obtained from animals treated with pilocarpine were also normal when examined by transmission electron microscopy (Fig. 2, *C*).

DISCUSSION

Pioneering work by Deitch et al.[14] showed that bacterial translocation is amplified whenever the intestinal epithelial layer is rendered discontinuous. Their studies used in vivo models of translocation such as hemorrhagic[15, 16] and endotoxic[17] shock and local (oral ricinoleic acid)[18] and systemic (intraperitoneal zymosan)[19] inflammation. The increased translocation under these experimental conditions may in part be due to decreased or altered mucus, as suggested by Deitch and Berg.[19]

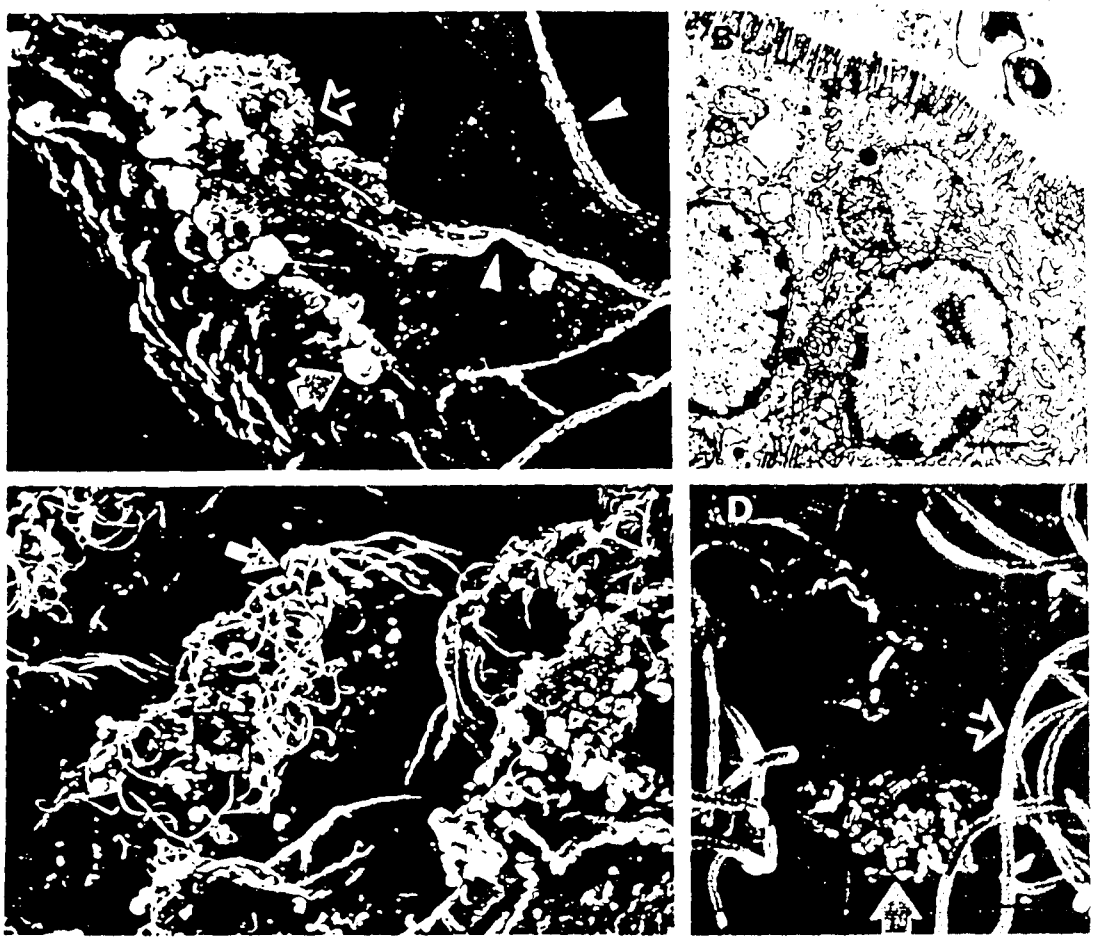

Fig. 2. A, Scanning electron micrograph of mucosal surface of control membrane after 180 minutes of perfusion in Ussing chamber. Normal surface epithelial structure is present with abundant strands and webs of mucus (*small arrows*) bridging the crypts. *Large arrow* shows ball of mucus that is not yet discharged from goblet cell. Clusters of bacteria are present (*clear arrow*). (Original magnification ×350.) B, Transmission electron micrograph of mucosal face of membrane from pilocarpine-treated animal after 180 minutes of perfusion in Ussing chamber. Surface structure is normal and underlying cytoarchitecture is well preserved. (Original magnification ×7250.) C, Scanning electron micrograph of mucosal surface of membrane from pilocarpine-treated animal after 180 minutes of perfusion in Ussing chamber. Epithelial surface structure is normal, but mucus is completely absent except for scattered unreleased mucus balls at tip of villi (*arrow*). Fewer bacteria are present on mucosal surface. (Original magnification ×350.) D, Higher magnification of *inset* from C. Bacillary microbes (*large arrow*) and filamentous organisms (*clear arrow*) indigenous to rat ileum are evident on epithelial cell surface that is devoid of mucus. These filamentous organisms have been described elsewhere,[13] and their presence has been confirmed in all of our ileal preparations. (Original magnification ×750.)

Our findings that depletion of intestinal mucus after intraperitoneal injection of pilocarpine increases bacterial passage across fresh perfused ileum support these data, although the models are not comparable.

For bacteria to penetrate the intestinal wall, they must first come in contact with that wall. Intestinal mucus is an epithelial secretion with special properties that should enable it to trap bacteria intraluminally and thereby inhibit binding to the intestinal lining. Indeed, large numbers of bacteria are found within mucus, whereas bacteria are relatively scare on the underlying mucosa and glands.[20] Intestinal mucus exists in two forms: a water-insoluble gel adherent to the mucosal epithelium and a water-soluble mucus within the lumen.[21] The adherent gel can act as a physical barrier by excluding molecules greater than 17,000 daltons.[22] Lack of exclusion of large molecules leading to translocation was shown by the increased permeability of the gut epithelium to horseradish peroxidase (molecular weight, 40,000 daltons) after experimental hemorrhagic shock.[16] Mucus can entrap bacteria either nonspecifically by virtue of its stickiness or specifically as bacte-

rial binding to its mucin oligosaccharides. The mucin receptors for bacterial adherence have been found to mimic epithelial cell surface receptors and therefore offer competitive attachment sites for lectins, toxins, and pathogens.[23-25] Several observations[26, 27] indicate that mucus may adhere to the epithelial cell surface with considerable tenacity. Consequently, bacteria may adhere in vivo through receptors within the mucus gel coating the cell surface rather than through cell receptors of the adjacent microvillus surface proper.

Mucus may also work synergistically with other antibacterial mechanisms. Lysozyme, a bacteriolytic enzyme, and lactoferrin, a bacteriostatic molecule, are well maintained within mucus.[22] Of even greater importance is the finding that secretory immunoglobulin A (sIgA), the predominant immunoglobulin of the gut barrier, binds the mucin glycoproteins through hydrogen or disulfide bonds in its hinge region.[28] This region of the sIgA molecule is structurally similar to mucin glycoproteins so that it is more soluble in mucus, suggesting a cooperative role between sIgA and mucus.

Mucus release from intestinal goblet cells is triggered by immune complexes, toxins, lectins, and cholinergic stimulation, all resulting in an increased net flow of mucus from epithelium to lumen.[7, 22] Specian and Neutra[7] demonstrated qualitative depletion of rat intestinal goblet cell mucus by use of light microscopy, electron microscopy, and autoradiography after in vivo pilocarpine administration. Based on their observations, one might expect that depletion of ileal mucus might increase bacterial attachment to the intestinal surface and thereby enhance translocation of bacteria through the membrane. Indeed, pilocarpine given to rats 45 minutes before intestinal harvest, a procedure known to deplete mucus, markedly increased the rate of translocation of *E. coli* C-25 from mucosal to submucosal surface in our fresh, perfused ileal segments. These studies are consistent with other investigations. Wells et al.[29] depleted intestinal mucus in vivo by feeding rats a crude red kidney bean extract that contained phytohemagglutinin, a lectin known to decrease intestinal mucus. They noted a significantly increased rate of translocation of *E. coli*, enterococci, and α-streptococci to mesenteric lymph nodes, liver, and spleen as compared with controls. Less mucus overlying ileal tissue was noted by transmission electron microscopy in treated animals and a significantly increased number of gram-negative and gram-positive bacteria cultured from segments of ileum.

One may speculate that the increased passage across pilocarpine-treated membranes resulted not solely from depleting mucus but from depleting compounds contained or concentrated within the mucus. For example, Wilson et al.[30] demonstrated by using in situ isolated perfused intestinal loops that cholinergic drugs (pilocarpine, bethanechol, and muscarine) increase baseline IgA secretion into the intestinal lumen and anticholinergic drugs decreased this secretion. Because we have shown that sIgA also impairs bacterial translocation, we cannot distinguish in our present experiments between the prophylactic effect as a result of the physical and chemical nature of the mucus itself or of its ligands. Regardless of mechanism, intestinal mucus appears to be a critical factor in the barrier function of the gut, and its depletion resulted in an increase in bacterial passage across the rat ileum.

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