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Role of Bacterial Adherence and the Mucus Barrier on Bacterial Translocation

Effects of Protein Malnutrition and Endotoxin in Rats

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Objective
The purpose of the study was to investigate the potential relations between mucosal bacterial adherence, intestinal mucus and mucin content, and bacterial translocation.

Summary Background Data
The attachment of bacteria to mucosal surfaces is the initial event in the pathogenesis of most bacterial infections that originate at mucosal surfaces, such as the gut. The intestinal mucus layer appears to function as a defensive barrier limiting micro-organisms present in the intestinal lumen from colonizing enterocytes. Consequently, studies focusing on the biology of bacterial adherence to the intestinal mucosa likely are to be important in clarifying the pathogenesis of gut origin sepsis.

Methods
To explore the relations between intestinal bacterial adherence, mucus bacterial binding, and bacterial translocation, two models were used. One (protein malnutrition) in which profound alterations in intestinal morphology occurs in the absence of significant translocation and one (endotoxin challenge) in which bacterial translocation occurs and intestinal morphology is relatively normal.

Results
Protein malnutrition was not associated with bacterial translocation and measurement of enteroadherent, mucosally associated bacterial population levels documented that the total number of gram-negative enteric bacilli adherent to the ileum and cecum was less in the protein-malnourished rats than in the normally nourished animals ($p < 0.01$). Furthermore, there was an inverse relation between the duration of protein malnutrition and bacterial adherence to the intestinal mucosa ($r = 0.62$, $p < 0.002$). In contrast, after endotoxin challenge, the level of enteroadherent bacteria was increased and bacterial translocation was observed. The binding of *Escherichia coli* to immobilized ileal mucus *in vitro* was decreased significantly in protein-malnourished rats, whereas *E. coli* binding to insoluble ileal mucus was increased in the rats receiving endotoxin.

Conclusions

This study indicates that the adherence of bacteria to the intestinal mucosal surface is an important factor in bacterial translocation, that intestinal mucus modulates bacterial adherence, and that increased levels of mucosally associated bacteria are associated with a loss intestinal barrier function to bacteria.

The intestinal mucosa functions as a major local defense barrier that helps to prevent the invasion and systemic spread of bacteria and endotoxin normally contained within the intestinal lumen. However, under certain conditions, intestinal mucosal barrier function appears to be impaired or overwhelmed, allowing indigenous bacteria or endotoxin within the gastrointestinal tract to reach systemic organs and tissues, a process termed bacterial translocation.[1] Because of the potential relation between loss of intestinal barrier function and the development of systemic infection or multiple organ failure or both in patients who are stressed and immunocompromised, numerous studies investigating this topic have been published over the past decade.[2] Although much remains to be learned of the exact mechanisms by which bacteria cross the intestinal mucosal barrier, as well as the relative importance of the various host defense factors that comprise the mucosal barrier, it is clear that the attachment of bacteria to mucosal surfaces is the initial event in the pathogenesis of most bacterial nosocomial infections that originate at mucosal surfaces.[3] Consequently, studies focusing on the biology of bacterial adherence to the intestinal mucosa likely are to be important.

The importance of the mucus layer as a defensive barrier that protects gastric and intestinal epithelial cells from luminal toxins such as acid, bile, and digestive enzymes is well recognized.[4,5] In addition, the mucus layer also appears to function as a barrier to enteric bacteria and their toxins. In fact, since the work of Florey[6] in 1933, it often has been claimed that the mucus layer acts as a mechanical barrier, restraining micro-organisms present in the intestinal lumen from reaching and colonizing the epithelial surface of the gut.[7] Additionally, there is abundant evidence that several strains of enteropathic *Escherichia coli,* as well as other pathogens, bind to the mucus gel better than nonpathogenic bacteria,[3,7] further indicating that the ability of bacteria to bind to mucus may be an important virulence factor. Likewise, studies of intestinal mucin may be important, because mucin is the major component of the mucus gel and it accounts for the viscosity and elasticity of this gel and provides binding sites for bacteria, immunoglobulins (particularly immunoglobulin A), and other local products.[4,5] Consequently, the major aim of this study was to investigate potential relations between mucosal bacterial adherence, intestinal mucus and mucin content, and bacterial translocation. To accomplish this aim, we used two models, one in which profound alterations in intestinal morphology occur in the absence of bacterial translocation (protein malnutrition [PM]) and one in which bacterial translocation occurs and intestinal morphology is relatively normal (endotoxin).[8,9]

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MATERIAL AND METHODS

Experimental Design

Specific pathogen-free male Sprague-Dawley rats, weighing 300 to 350 gm, were housed under barrier-sustained conditions and maintained in accordance with the recommendations of the National Research Councils Guide for the Care and Use of Laboratory Animals. The experiments were approved by the Louisiana State University-Medical Center Shreveport and The UMDNJ-New Jersey Medical School Newark Animal Care Committees.

The rats were fed standard laboratory chow (Diet 5001, Ralston Purina, St. Louis, MO) and acidified water (0.001 N hydrochloric acid) *ad libitum.* This standard rat diet contains 17% protein and 11% fat by weight. In the protein-depletion experiments, the rats received a low-protein, solid-whey diet (Tekland Test Diets, Madison, WI) for periods up to 21 days. This diet meets the requirements of the National Research Council for trace elements, vitamins, and minerals and contains 20% fat, 67% carbohydrates, but only 0.03% protein by weight, rendering it nearly protein free. Animals in both groups received drinking water *ad libitum.* Lipopolysaccharide (endotoxin) from *E. coli* 0127:B8 (Sigma Chemical, St. Louis, MO) was dissolved in normal saline to a final concentration of 5 mg/mL. After 0, 6, 13, or 20 days of PM, 100 mL (0.5 mg) of endotoxin per 100 g body weight was injected intraperitoneally into each rat of the endotoxin groups, whereas the control rats were injected with saline. Twenty-four hours later, after an overnight fast, the animals were weighed and then killed. The mesenteric lymph node (MLN) was excised and quantitatively cultured to assess the effect of PM only, endotoxin only, or the combination on bacterial translocation. After removing the

MLN, the cecum and the small intestine, from the ligament of Treitz to the ileocecal valve, were excised. The length and weight of the small bowel was recorded. The distal 4- to 5-cm region of the ileum (distal ileum) was removed. Each cecal and distal ileal segment was cut in two. One half was quantitatively cultured without further preparation to measure total bacterial population levels, whereas the second half was processed to remove nonadherent bacteria before culturing, thereby allowing only enteroadherent bacterial population levels to be measured. The remaining small bowel (after the distal ileal segment was removed) was divided into three pieces consisting of proximal, middle, and distal small intestine. Each segment was washed and the intestinal washings from these small bowel segments were collected for measurement of luminal mucus and mucin (soluble) levels. Thereafter, the washed small intestinal segments were homogenized and processed for mucosal protein and insoluble mucus and mucin content. Lastly, using an *in vitro* adhesion assay, the ability of luminal contents (soluble mucin) and tissue (insoluble) mucin from the various groups of rats to bind radiolabeled bacteria was measured.

Testing for Bacterial Translocation, Cecal, and Ileal Population Levels of Bacteria

The MLN complex was tested for translocating bacteria as described previously.[8,9] Using sterile technique, the MLN complex was weighed and homogenized, and aliquots (0.2 mL) were plated onto blood and MacConkey agar plates. Based on the weight of the MLN and the dilutions used, the limit of detection of bacteria translocating to the MLN was 50 colony-forming units (CFU)/g MLN (1.7 $\log_{10}$ CFU/g MLN). The plates were examined after 24 and 48 hours of aerobic incubation at 37 C.

After the MLN had been removed for culturing of translocating bacteria, the cecum and a 5-cm segment of distal ileum were removed. The luminal contents from the distal ileal segment of the small intestine were collected. Subsequently, the cecum and distal ileum were cut in half, and each half was weighed. One half of each specimen was homogenized immediately, and serial dilutions of the homogenate of cecum, distal ileum, and luminal contents were plated onto blood and MacConkey agar plates to measure total intestinal bacterial population levels. The other half of the cecum and distal ileum was processed using a modification of the method described by Alverdy and Aoys.[10] to quantitate mucosal-associated intestinal bacterial population levels. In this assay, the cecal and distal ileal specimens were placed into tubes containing saline and vortexed vigorously for 3 seconds. The intestinal samples then were removed and placed into tubes containing fresh saline and vortexed again. After this vortexing process was repeated three times to remove all nonmucosally associated bacteria from the intestinal segments, the tissues were homogenized and serial dilutions of the cecal and distal ileal homogenates were plated onto blood and MacConkey agar plates. All plates were examined after incubation for 24 and 48 hours at 37 C. Adherent and total bacterial population levels were expressed as $\log_{10}$ CFU/g of cecum or $\log_{10}$ CFU/cm of ileum. Gram-negative enteric bacilli and gram-positive cocci were identified by standard procedures as described previously.[8]

We measured levels of mucosally associated, as well as total intestinal, bacteria in the cecum and ileum, because previously we documented that endotoxin preferentially damaged the cecal and ileal mucosa and increased the permeability of the ileum more than the jejunum.[11]

Preparation of Rat Small Intestinal Mucus

The abdomen was opened and the entire small intestine, from the ligament of Treitz to the cecum, was removed and transferred to a dish containing ice-cold phosphate-buffered saline (PBS). After the distal 5 cm of ileum was excised, the remaining small intestine was divided into equal thirds. The exact length of the distal ileum and three small bowel segments was determined under vertical extension with a 1.5-g weight.

Intestinal mucus consists of two components; 1) a water-soluble component (soluble mucus), which is present in the lumen and 2) a water-insoluble component (insoluble mucus), which is present in goblet cells and as an adherent gel bound to the mucosal surface.[4,5] To obtain soluble mucus, each of the small bowel segments was squeezed gently and then the lumen was flushed with 1 mL of ice-cold PBS, pH 7.2. The luminal contents of these segments were collected on ice. The luminal contents then were homogenized for 30 seconds and washed twice at 3000 revolutions per minute for 10 minutes,[12] after which the supernatants were harvested and stored at −70 C in preparation for quantitating soluble mucin levels. Thereafter, the washed small intestines were processed for insoluble mucus and mucin content as follows. The intestine was opened longitudinally on ice. The mucosal layer gently was scraped off with a rubber spatula, weighed, and transferred to a separate tube containing 1 mL of ice-cold PBS. The weight of each scraped segment of small intestine was recorded. Tissue (insoluble) mucus was homogenized for 30 seconds and centrifuged at 28,000 × g for 15 minutes to remove cellular material and any remaining fecal debris.[12] The supernatants containing the insoluble mucin fraction were collected and stored at −70 C.

Mucin Assay

An indirect enzyme-linked immunosorbent assay was developed after the methods of Mantle et al.,[13] with a

standard mucin purified from rat mucus and an antimucin antibody raised against that standard in New Zealand white rabbits.[14] Briefly, a 96-well flat bottom microtiter plate was coated overnight at 4 C with 500 ng of purified mucin standard in 50 mL of PBS. In a second plate, 50 mL of the test samples or 50 mL of the mucin standards (range, 0 to 4000 ng) was preincubated with 75 mL of antimucin antibody (1:1000 dilution) at 37 C. After the incubation period, the mucin-coated plate was washed twice with wash buffer (0.05% Tween 20, 0.1-M Tris, 0.15-M sodium chloride, pH 8). The wells were blocked with 150 mL of 5% bovine serum albumin in PBS for 1 hour at room temperature and washed twice with wash buffer.

Fifty microliters of each preincubated sample and standard were transferred to wells of the mucin-coated microtiter plate. After a 1-hour incubation at room temperature, each well was washed three times with the wash buffer. Fifty microliters containing 50 mg of horseradish peroxidase conjugated protein A (1:1000 dilution in PBS, Bio Rad, New York) was added to each well and allowed to bind to the antigen-bound antibody for 1 hour at room temperature. Then, each well was washed three times with wash buffer to remove all bound enzyme. The substrate (150 mL of 8.8-mmol hydrogen peroxide and 0.6% orthophenylenediamine in citrate PBS, pH 5.5) then was added to each well. After 30 minutes in a dark room, the reaction was terminated with 50 mL of 2-N hydrochloric acid and the absorbance quantitated at 490 nm. All experiments were performed in duplicate. Standard curves were constructed by plotting the absorbance in the well (linear ordinate) against the amount of standard antigen in preincubation mix (logarithmic abscissa). The mucin levels were normalized for the length of the respective intestinal segment and for the mucosal protein content.

Mucosal Protein

The scraped mucosal samples were homogenized for 30 seconds. The homogenates were centrifuged and the supernatant was assayed spectrophotometrically (596 nm) for protein by the Coomassie blue method (Bio-Rad Laboratories, Richmond, CA).[15] Bovine serum albumin was used as the standard. Mucosal protein content was expressed as milligrams of protein per centimeter of gut length to correct for differences in gut length between animals.

Bacterial Growth Conditions and Radiolabeling of Bacteria

E. coli C25 were grown in sterile minimum essential medium containing 10% fetal serum albumin to which 100 mCi of sterile [^{3}H] thymidine had been added. After 20 hours of incubation at 37 C, the bacteria were harvested by centrifugation at 2800 g and washed twice. Bacteria were suspended in Hepes–Hanks buffer to a concentration of approximately 1×10^9/mL. Radiolabeling resulted in approximately 5×10^3 bacteria/counts per minute.

Adhesion Assay *In Vitro*

A modification of the method described by Laux et al.[12] was used to study adherence of *E. coli* to mucus. Assays were performed in 96-well polystyrene tissue culture plates (Costar, Cambridge, MA). Different preparations of mucus or luminal contents (0.1 mg of protein in 0.1 mL) were incubated overnight in wells at 4 C. Unbound protein was removed by three washes of Hepes–Hanks buffer (pH 7.2), and the coated wells were stored at −20 C until assayed. To block residual binding sites, 0.15 mL of 5% bovine serum albumin was added to the wells for 2 hours at 25 C. Unbound bovine serum albumin then was removed by washing the wells three times with Hepes–Hanks buffer (pH 7.2). All experiments were performed in duplicate. Radiolabeled *E. coli* (0.1 mL, 1×10^9 CFU/mL, 1.5×10^5 counts per minute/mL) were added to each well and incubated for 2 hours at 37 C and then washed three times with Hepes–Hanks buffer (pH 5.0). Adherent bacteria were recovered from the wells by adding 0.15 mL of 5% sodium dodecyl sulfate to each well and reincubating the plates at 37 C for 3 hours. After this incubation period, the contents of each well were harvested and the level of radioactivity determined by liquid scintillation counting.

E. Coli Growth Assay in Tissue and Luminal Contents

The effect of tissue mucus and luminal contents on bacterial growth was assessed as follows. The streptomycin-resistant *E. coli* C25 strain (10^4 CFU) was incubated in 0.45 mL of tissue mucus (4 mg of protein/mL) or luminal contents (1 mg of protein/mL) at 37 C. After 0, 4, and 20 hours, 0.1-mL samples were removed, serially diluted, and plated on MacConkey agar plates containing streptomycin. *E. coli* C25 growth on the MacConkey agar plates was quantitated after incubation at 37 C for 18 hours.

Statistical Analysis

Translocation incidences (discontinuous data) were evaluated by chi square analysis with the Yates correction. Continuous data were analyzed by analysis of variance using the *post hoc* Neumann–Keuls test or by Student's t test as appropriate. Correlations between variables were

Table 1. PROTEIN MALNUTRITION (MLN) INCREASES THE INCIDENCE OF ENDOTOXIN-INDUCED BACTERIAL TRANSLOCATION (BT)

	n	BT to MLN (%)	BT to MLN (log 10 CFU/g)	
			Total Aerobes	Gram-Negative Enterics
PM group				
Control	10	0	1.70 ± 0	1.70 ± 0
PM7 day	10	10	1.88 ± 0.56	1.84 ± 0.44
PM14 day	8	0	1.7 ± 0	1.70 ± 0
PM21 day	10	30	2.06 ± 0.56	1.99 ± 0.44
Endotoxin group				
Control + endotoxin	8	62.5*	2.36 ± 0.55*	2.19 ± 0.46
PM7 day ± endotoxin	8	82.5*	2.73 ± 0.60*	2.59 ± 0.50*
PM14 day ± endotoxin	8	82.5*	2.76 ± 0.64*	2.60 ± 0.62*
PM21 day + endotoxin	8	100*	3.40 ± 0.50*†	3.21 ± 0.60*†

* $p < 0.01$ *vs.* PM group at the same time.
† $p < 0.05$ *vs.* control and PM7 day + endotoxin.
Values are mean ± SD.

determined by linear regression analysis. Probabilities less than 0.05 were considered significant.

RESULTS

As reported previously,[8,9] bacterial translocation did not occur consistently in the normally nourished or protein-malnourished rats unless they were challenged with endotoxin (Table 1).

The cecal and ileal bacterial population levels of the protein-malnourished rats minimally were effected by the low-protein diet (Table 2). In contrast, the cecal and ileal bacterial population levels were 100 to 1000-fold higher in the endotoxin-treated than in the nonendotoxin-treated animals (Table 2), verifying previous studies indicating that endotoxin disrupts the normal intestinal flora.[8,11] Measurement of enteroadherent, mucosally associated bacterial population levels documented that the total numbers of aerobic, as well as gram-negative, enteric bacilli adherent to the ileum and cecum were less in the protein-malnourished rats than in the normally nourished animals (Table 3). Similar to that observation, the levels of enteroadherent cecal and ileal bacteria were higher in the endotoxin-treated than in the nontreated rats at all time points (Table 3). Because the levels of enteroadherent bacteria in the endotoxin-treated, normally nourished rats were not higher than those of the protein-malnourished rats receiving endotoxin, it appears that the PM-mediated decrease in adherent bacterial populations fully was reversible by endotoxin.

Furthermore, there was an inverse relation between the duration of PM and the number of bacteria adherent to the ileal mucosa (total aerobes; $R = 0.68$, $p = 0.000003$, gram-negative enterics; $R = 0.66$, $p = 0.000006$) (Fig. 1). A similar inverse correlation was observed in the cecum (data not shown). Although, in general, the number of enteroadherent bacteria increased over time in the rats receiving endotoxin, these differences did not reach statistical significance.

To examine whether the modulatory effects of PM and endotoxin on the numbers of enteroadherent gram-negative enteric bacteria was related to changes in mucus-bacterial binding, we measured the mucus-binding capacity of ileal tissue mucus and luminal content samples for *E. coli* C25 *in vitro* (Table 4). The binding of *E. coli* C25 to immobilized insoluble ileal mucus was decreased significantly in the protein-malnourished rats not receiving endotoxin, whereas PM had no effect on the binding of *E. coli* to partially purified ileal luminal soluble mucus. In contrast, *E. coli* binding to insoluble ileal mucus and ileal luminal-soluble mucus was increased in the rats receiving endotoxin (Table 4).

Because mucus can be a substrate for bacterial growth,[5] we investigated whether there was a difference in the ability of ileal-soluble or -insoluble mucus from the different experimental groups to support the growth of *E. coli* C25 *in vitro* (Fig. 2). Because there was no difference in *E. coli* C25 growth among the groups, the differential effects of PM and endotoxin on the levels of enteroadherent bacteria observed *in vivo* do not appear to be directly related to differences in the ability of mucus from these groups to support the growth of bacteria.

The administration of the low-protein diet was associated with a decrease in small intestinal weight in both the rats receiving and not receiving endotoxin, although the mucosal protein content per centimeter of intestine did

Table 2. THE EFFECT OF PROTEIN MALNUTRITION WITH OR WITHOUT ENDOTOXIN ON BACTERIAL POPULATION LEVELS IN THE CECUM AND ILEUM

		Ileal Population Levels (log 10 CFU/g)		Cecal Population Levels (log 10 CFU/g)	
	n	Total Aerobes	Gram-Negative Enterics	Total Aerobes	Gram-Negative Enterics
PM group					
Control	10	5.5 ± 0.7	3.8 ± 0.5	7.2 ± 1.0†	6.3 ± 0.5
PM7 DAY	10	4.3 ± 1.1	3.1 ± 1.1	6.6 ± 0.3	6.2 ± 0.2
PM14 day	8	4.6 ± 0.8	4.0 ± 0.6	6.5 ± 0.4	5.9 ± 0.6
PM21 day	10	4.6 ± 0.8	3.3 ± 1.1	6.1 ± 0.4	5.9 ± 0.5
Endotoxin group					
Control + endotoxin	8	6.8 + 1.0*	5.7 + 1.1*	9.0 + 0.7*	8.3 + 1.0*
PM7 day + endotoxin	8	6.7 + 1.5*	6.5 + 1.5*	8.9 + 0.7*	8.7 + 0.7*
PM14 day + endotoxin	8	6.5 + 1.2*	6.2 + 1.3*	8.8 + 1.0*	8.6 + 1.1*
PM21 day + endotoxin	8	7.0 + 1.1*	6.0 + 1.1*	9.2 + 0.8*	9.2 + 0.4*

* $p < 0.01$ *vs.* PM group at the same time.
† $p < 0.01$ *vs.* other PM groups.
PM = protein malnutrition.
Values are mean + SD.

not change (Table 5). Intestinal insoluble mucin levels were decreased significantly after 7, 14, or 21 days of PM, whether expressed as micrograms mucin per centimeter of intestine or micrograms mucin per milligram of mucosal protein (Table 6). Although endotoxin administration was associated with a decrease in insoluble mucin levels in the normally nourished rats, the levels of insoluble mucin were similar between the protein-malnourished rats receiving and not receiving endotoxin (Table 6). In contrast, although the soluble mucin levels in the luminal contents of the upper intestine were increased in the 14- and 21-day protein-malnourished animals receiving endotoxin, overall soluble mucin levels were effected minimally by PM or endotoxin administration (Table 7).

DISCUSSION

In previous studies, we found that PM alone did not promote bacterial translocation, although the protein-mal-

Table 3. ENTEROADHERENT BACTERIAL POPULATION LEVELS OF ILEAL MUCOSA AND CECAL MUCOSA

		Adherent Bacteria to Ileum (log CFU/g)		Adherent Bacteria to Cecum (log CFU/g)	
	n	Total Aerobes	Gram-Negative Enterics	Total Aerobes	Gram-Negative Enterics
PM group					
Control	10	4.3 ± 1.1	3.7 ± 1.5	5.6 ± 1.0	4.5 ± 1.0
PM7 day	10	3.2 ± 1.0*	1.8 ± 1.3*	5.1 ± 1.0*	4.2 ± 0.7*
PM14 day	8	2.2 ± 1.6*	0.9 ± 1.7*	4.6 ± 0.5*	3.3 ± 0.9†
PM21 day	10	1.4 ± 1.0*	0.5 ± 0.7*	4.4 ± 0.5*	2.6 ± 0.9*
Endotoxin group					
Control + endotoxin	8	5.7 ± 1.0†	4.5 ± 0.6	7.2 ± 0.6§	6.6 ± 0.7§
PM7 day + endotoxin	8	5.5 ± 1.1§	5.3 ± 1.0§	7.3 ± 0.8§	7.0 ± 0.8§
PM14 day + endotoxin	8	6.2 ± 1.1§	5.8 ± 1.0§	7.2 ± 1.4§	7.1 ± 0.8§
PM21 day + endotoxin	8	6.3 ± 0.8§	5.2 ± 1.1§	7.8 ± 0.5§	7.3 ± 0.6§

* $p < 0.01$ *vs.* control.
† $p < 0.05$ *vs.* control.
‡ $p < 0.05$ *vs.* PM group at the same time.
§ $p < 0.01$ *vs.* PM group at the same time.
PM = protein malnutrition.
Values are mean ± SD.

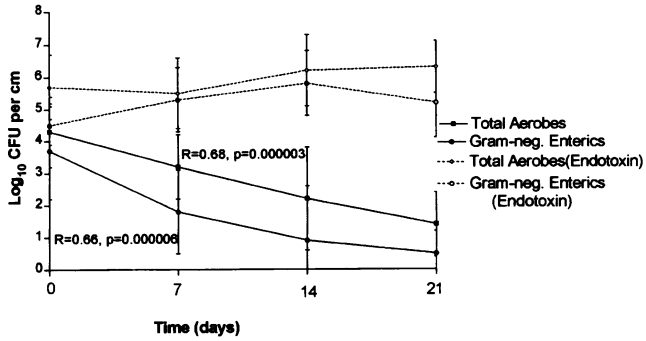

Figure 1. An inverse relation between the duration of protein malnutrition and the number of bacteria adherent to the ileal mucosa (total aerobes, R = 0.68; p = 0.000003; gram negative enterics, R = 0.66, p = 0.000006).

nourished rats were more susceptible to endotoxin-induced bacterial translocation, and both the magnitude of bacterial translocation and the mortality rate after endotoxin challenge were related directly to the degree of PM. The fact that bacterial translocation did not occur in the protein-malnourished animals unless they were further challenged with endotoxin was puzzling, because the protein-malnourished animals lost significant amounts of their body weight, had severe mucosal atrophy develop,[8,9] and were immunocompromised.[16] In fact, these results suggested that mucosal atrophy and intestinal barrier function are not necessarily related directly and that other factors besides the intestinal mucosa may be involved in

Table 4. ADHERENCE OF *E. COLI* C25 TO IMMOBILIZED INSOLUBLE ILEAL MUCUS OR SOLUBLE LUMINAL MUCUS

	n	Adherent Bacteria (cpm/well)	
		Insoluble Mucus	Soluble Mucus
PM group			
Control	8	1067 ± 365*	326 ± 74
PM7 day	8	491 ± 221	385 ± 150
PM14 day	6	481 ± 194	411 ± 78
PM21 day	8	377 ± 87	302 ± 42
Endotoxin group			
Control + endotoxin	6	1411 ± 377	981 ± 179†
PM7 day + endotoxin	6	1352 ± 363†	931 ± 502‡
PM14 day ± endotoxin	6	1605 ± 365†	793 ± 277‡
PM21 day ± endotoxin	6	1152 ± 396†	732 ± 173†

* $p < 0.01$ *vs.* other PM groups.
† $p < 0.01$ *vs.* PM group at the same time.
‡ $p < 0.05$ *vs.* PM group at the same time.
PM = protein malnutrition. Values are mean ± SD.

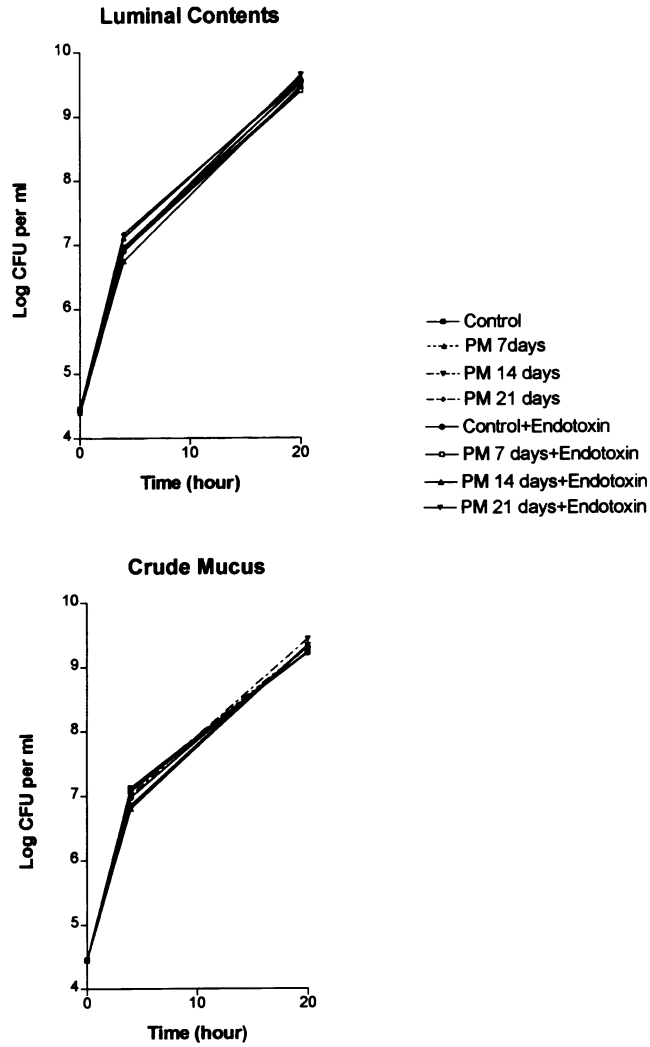

Figure 2. No differences are noted in the ability of ileal-soluble or -insoluble mucus from the different experimental groups to support growth of *Escherichia coli* C25 *in vitro*.

biologic intestinal barrier function. Because the functional mucosal barrier consists of many factors,[2] one of the most important of which is the mucus layer, the major goal of the current study was to investigate potential relations between mucosal bacterial adherence, intestinal mucus, and mucin content and bacterial translocation. In that light, the current observation that PM is associated with a significant decrease in mucosal-associated, enteroadherent intestinal bacterial population levels helps explain why bacterial translocation rarely occurred in the protein-malnourished animals. Likewise, the fact that endotoxin increased mucosal-associated bacterial levels in both normally nourished and protein-malnourished rats helps explain the ability of endotoxin to promote bacterial translocation.

The fact that mucosal-associated but not total (luminal

Table 5. SMALL INTESTINAL WEIGHT AND MUCOSAL PROTEIN CONTENT

	Weight of Small Intestine (g/cm)	Mucosal Protein Content of Small Intestine (mg/cm)
PM group		
Control	48.1 ± 4.0*	0.50 ± 0.06
PM7 day	38.3 ± 2.8*	0.62 ± 0.08
PM14 day	33.8 ± 4.2	0.51 ± 0.10
PM21 day	31.4 ± 1.9	0.57 ± 0.09
Endotoxin group		
Control + endotoxin	42.3 ± 4.9*†	0.49 ± 0.13
PM7 day + endotoxin	35.1 ± 4.2	0.55 ± 0.06
PM14 day + endotoxin	34.6 ± 3.1	0.51 ± 0.06
PM21 day + endotoxin	30.5 ± 3.7	0.45 ± 0.22

* $p < 0.01$ *vs.* other PM groups.
† $p < 0.05$ *vs.* PM only group at the same time.
Values are mean + SD (upper, mid, and distal intestinal values combined).

plus mucosal-associated) intestinal bacterial levels were reduced in the protein-malnourished rats highlights the importance of recognizing that there are two distinct intestinal microfloras in rats as well as humans and other mammals.[17] The first intestinal microflora is found within the lumen of the gut, and the second is associated with the mucosal surface. Although the levels of luminal bacteria clearly are important in the development of infection after bowel resection or when the bowel is injured or perforated, the levels of mucosal-associated bacteria likely are to be more important in the development of bacterial translocation.

There are several potential reasons why mucosal-associated bacterial levels were reduced in the protein-malnourished rats. For example, the decrease in mucosal-associated bacterial levels could be related to the decrease in mucosal surface area due to mucosal atrophy. However, this explanation is not likely, because the levels of mucosal-associated bacteria were increased in the protein-malnourished rats receiving endotoxin, and the degree of mucosal atrophy was similar in the protein-malnourished rats receiving and not receiving endotoxin.[9] A second possibility is that the effects of PM or endotoxin or both on the mucosal-associated bacterial population levels were related to the ability of the intestinal mucus to support or inhibit bacterial growth. This does not appear to be the case, because intestinal mucus harvested from the protein-malnourished as well as the endotoxin-treated rats supported the growth of *E. coli* as well as intestinal mucus harvested from normally nourished rats. Lastly, the decrease in mucosal-associated bacterial levels in the protein-malnourished rats could be because of reduced bacterial adherence to the intestinal mucus layer. This explanation is supported by our observation that *E. coli* bound less avidly to mucus from the protein-malnourished rats than the normally nourished rats and that endotoxin treatment reversed this effect. These results indicate that the alterations in the levels of mucosal-associated bacteria observed in the current study might be related to changes in the ability of bacteria to bind to the intestinal mucus layer.

Other studies have been published recently investigating the effects of mucus on intestinal permeability and bacterial translocation.[14,18–21] Two reports[14,18] documented that parenteral alimentation was associated with a de-

Table 6. INSOLUBLE MUCIN LEVELS IN HOMOGENATES OF UPPER, MID, AND DISTAL SMALL INTESTINAL MUCOSA

		Insoluble Mucin per Gut Length (μg/cm)				Insoluble Mucin/Mucosal Protein (μg/mg)			
	n	Upper	Mid	Distal	Total	Upper	Mid	Distal	Total
PM group									
Control	10	64 ± 31*	88 ± 39*	91 ± 48*	79 ± 32*	126 ± 61*	170 ± 62*	181 ± 77*	157 ± 53*
PM7 day	8	30 ± 8	50 ± 18	39 ± 7	40 ± 6	46 ± 11	75 ± 22	78 ± 30	66 ± 17
PM14 day	8	22 ± 14	37 ± 16	27 ± 18	32 ± 17	48 ± 15	70 ± 18	67 ± 30	63 ± 17
PM21 day	10	28 ± 10	40 ± 14	29 ± 10	32 ± 6	48 ± 22	69 ± 26	56 ± 14	58 ± 12
Endotoxin group									
Control + endotoxin	8	25 ± 7†	51 ± 43	25 ± 12†	34 ± 16†	42 ± 8‡	105 ± 71	66 ± 41†	71 ± 36†
PM7 day + endotoxin	8	22 ± 11	34 ± 16	31 ± 12	29 ± 9‡	40 ± 20	61 ± 27	58 ± 23	53 ± 16
PM14 day + endotoxin	8	28 ± 10	37 ± 14	36 ± 18	33 ± 11	57 ± 27	81 ± 60	87 ± 52	75 ± 42
PM21 day + endotoxin	8	16 ± 6‡	30 ± 12	35 ± 23	27 ± 10	42 ± 18	84 ± 59	97 ± 84	74 ± 50

* $p < 0.01$ *vs.* other PM groups.
† $p < 0.01$ *vs.* PM group at the same time.
‡ $p < 0.05$ *vs.* PM group at the same time.
Values are mean ± SD.

Table 7. MUCIN LEVELS IN LUMINAL CONTENTS FROM THE UPPER, MID, AND DISTALL SMALL INTESTINE

	n	Soluble Mucin (μg/cm)			
		Upper	Mid	Distal	Total
PM group					
Control	10	26 ± 26	36 ± 19	30 ± 16	30 ± 12
PM7 day	8	17 ± 14	25 ± 16	18 ± 10	20 ± 11
PM14 day	8	13 ± 7	24 ± 18	18 ± 11	20 ± 12†
PM21 day	10	16 ± 8	37 ± 23	28 ± 16	27 ± 13
Endotoxin group					
Control + endotoxin	8	31 ± 16	41 ± 13	37 ± 15	36 ± 11
PM7 day + endotoxin	8	17 ± 14	29 ± 19	32 ± 12*	24 ± 11
PM14 day + endotoxin	8	27 ± 14*	30 ± 13	30 ± 16	29 ± 9
PM21 day + endotoxin	8	36 ± 27*	38 ± 28	65 ± 66	46 ± 36

* $p < 0.05$ *vs.* PM group at the same time.
† $p < 0.05$ *vs.* control.
Values are mean ± SD (μg of mucin per cm of gut length).

crease in intestinal mucus content as well as an increase in intestinal permeability or bacterial translocation. Two additional studies have documented that drug-induced depletion of intestinal mucus will increase the rate of bacterial translocation across the ileal mucosa of otherwise normal rats.[19,20] Using a cell culture model, the last study showed that the presence of mucin will reduce the incidence and magnitude of bacterial passage across an intact enterocyte monolayer.[21] Taken together, these studies indicate that a decrease in intestinal mucus will potentiate bacterial translocation and support the concept that the mucus layer contributes to intestinal barrier function. Thus, these studies are consistent with our observation that intestinal mucin levels were decreased significantly after endotoxin challenge. However, the fact that intestinal mucin levels were as equally decreased in the protein-malnourished rats as in the endotoxin rats highlights the limitations of pure quantitative studies of intestinal mucus and indicates that qualitative as well as quantitative changes of intestinal mucus may modulate mucosal bacterial adherence. In fact, there is abundant evidence that intestinal mucins are heterogenous, and at least six different mucin species have been isolated from human and rat intestines.[4,5] Because different mucin species have different chemical and binding properties, alterations in mucin and mucus composition as well as changes in mucus content are likely to be important biologically. Further study will be required to address this issue.

Nonetheless, it is of interest that in an earlier study[22] using this same model of PM, we found that protein-malnourished mice were more resistant to colonization with exogenous *E. coli* than were normally nourished animals. Additionally, the increased colonization resistance associated with the protein-malnourished state appeared to prevent bacterial translocation in a bacterial overgrowth model of bacterial translocation. This protective effect of PM against bacterial overgrowth-induced bacterial translocation was in direct contrast to that observed in endotoxin- or inflammation-induced bacterial translocation, where the protein-malnourished animals were more susceptible to bacterial translocation.[8] The divergent effects of PM in these different models of bacterial translocation[8,22] highlight the limitations of generalizations made from the results of any one model or group of experiments and underline the complexity of biologic systems.

In summary, the biology of bacterial adherence to mucosal surfaces has received increasing attention in recent years, because the ability of bacteria to reach and adhere to mucosal surfaces has been shown to be an essential step in the pathogenesis of naturally occurring infections originating at mucosal surfaces. Furthermore, because the mucus gel is involved in bacterial adherence and bacterial adherence to the enterocyte appears to be an essential step in nutrition-induced bacterial translocation, factors that decrease the ability of bacteria to associate with the intestinal mucosa or bind directly to the intestinal epithelium would be expected to protect against bacterial translocation. Thus, the decrease of adherent bacteria to the mucosal surface might be one reason why severe PM did not promote bacterial translocation despite profound villous atrophy and significant suppression of immune defenses. Consequently, the results of this study indicate that the adherence of bacteria to the intestinal mucosal surface is an important component in bacterial translocation and that increased levels of mucosally associated

bacteria are associated with loss of normal mucosal barrier function.

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