Untreated Diabetes Mellitus Promotes Intestinal Microbial Overgrowth

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Gastrointestinal dysfunction is a common secondary complication of insulin-dependent diabetes mellitus, yet its etiology is unclear. Enteric microbial overgrowth may play a role. To quantitate the changes in mucosal-adherent enteric microbial populations in untreated diabetes mellitus and to assess the impact of two forms of insulin replacement therapy upon enteric microbial populations, age-matched male Lewis rats were rendered diabetic by the administration of intravenous streptozotocin (55 mg/kg). After diabetes was confirmed (blood glucose level greater than 250 mg/dL), rats were divided into three groups: no treatment (no insulin), treatment with daily insulin to maintain normoglycemia (3 to 7 units of protamine zinc insulin subcutaneously), or transplantation with a vascularized heterotopic duct-ligated pancreatic isograft. After 1 month, rats were killed, and segments of the proximal, middle, and distal small bowel were obtained. Mucosal samples were rinsed in phosphate-buffered saline to remove nonadherent bacteria prior to aerobic and anaerobic culturing. Microbial recovery was expressed as the $\log_{10}$ colony-forming unit/mg tissue wet weight. Untreated diabetes resulted in an overgrowth of mucosal-associated small bowel aerobic and anaerobic microbial populations compared with populations in normal nondiabetic age-matched control rats. Insulin treatment and pancreatic transplantation prevented microbial overgrowth in the diabetic small intestine. Pancreatic transplantation resulted in strict normoglycemia equivalent to that in nondiabetic control rats, whereas insulin treatment resulted in slightly higher blood glucose levels at sacrifice and wide fluctuations in blood glucose levels compared with nondiabetic control rats. These data suggest that sustained normalization of glucose levels is not required to prevent microbial overgrowth in diabetic rats.

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Patients with long-standing insulin-dependent diabetes mellitus experience significant morbidity and mortality due to the secondary complications of nephropathy, neuropathy, vasculopathy, and retinopathy. Gastrointestinal dysfunction, especially diarrhea, is a common secondary complication of insulin-dependent diabetes mellitus, yet its etiology is unclear. In patients with diabetes, diarrhea may result from an overgrowth of intestinal bacteria. Bacterial overgrowth may lead to diarrhea through such mechanisms as deconjugation of bile salts or production of hydroxylated fatty acids or by direct toxic effects upon the bowel mucosa. Bacterial overgrowth may be promoted either by diabetic autonomic neuropathy involving the gastrointestinal tract with impaired intestinal peristalsis or by altered substrate availability.

The goals of the current study were twofold. First, we sought to clearly define the alterations in mucosally adherent aerobic and anaerobic microbial populations in the small intestine in a rat model of chemically induced untreated diabetes. Second, we examined the impact of two forms of insulin replacement therapy, i.e., exogenous insulin and pancreatic transplantation, upon microbial populations in the small intestine. The use of exogenous insulin obviously mimics the clinical situation. Recent improvements in pancreatic allograft survival have led to greater acceptance of this therapy in selected patients with diabetes. Although the stated rationale of pancreatic transplantation is the prevention or retardation of secondary complications of diabetes by re-establishment of normal glucose homeostasis, its impact upon gastrointestinal dysfunction is unknown.

MATERIAL AND METHODS

Animals: Male Lewis rats (Harlan-Sprague-Dawley, Indianapolis, IN) weighing 250 to 275 g were rendered diabetic by the administration of intravenous streptozotocin (55 mg/kg). Streptozotocin is a selective β-cell toxin that causes hyperglycemia 24 hours after administration [*1*]. Diabetes was confirmed by two consecutive daily blood glucose levels greater than 250 mg/dL. All animals were maintained on standard rat chow and allowed water *ad libitum*. Within 4 days after injection of streptozotocin, diabetic animals were divided into three groups: untreated animals (no insulin), treated animals (3 to 7 units of protamine zinc insulin subcutaneously daily to maintain normoglycemia), and transplant animals (pancreatic isograft). Age-matched nondiabetic male Lewis rats were used as the control subjects. Blood glucose levels were recorded daily and prior to the time that the animals were killed.

TABLE I
Total Gram-Positive and Gram-Negative Aerobic and Anaerobic Microbial Recovery of Mucosally Adherent Flora in the Proximal Small Intestine*

Group	(Log_{10} CFU/mg Wet Weight [Mean ± SE])					
	Aerobes			Anaerobes		
	Total	Gram (+)	Gram (−)	Total	Gram (+)	Gram (−)
Control (n = 5)	4.9 ± 0.2	4.4 ± 0.3	3.2 ± 0.1	4.3 ± 0.4	4.5 ± 0.3	3.6 ± 0.4
Untreated (n = 5)	6.4 ± 0.2†	6.2 ± 0.6†	5.6 ± 0.2‡	6.6 ± 0.0†	6.5 ± 0.1†	5.9 ± 0.1†
Insulin (n = 5)	4.4 ± 0.3	4.2 ± 0.2	4.5 ± 0.0	3.9 ± 0.3	3.7 ± 0.4	3.0 ± 0.4
Pancreas transplant (n = 4)	4.5 ± 0.1	4.3 ± 0.1	3.6 ± 0.6	3.1 ± 0.3	2.9 ± 0.5	2.2 ± 0.1

*Untreated diabetes resulted in aerobic and anaerobic microbial overgrowth, whereas insulin replacement therapy normalized microbial overgrowth.
†$p < 0.05$ versus control, insulin, and transplant groups by ANOVA and Scheffe's test.
‡$p < 0.05$ versus control and transplant groups by ANOVA and Scheffe's test.

TABLE II
Total Gram-Positive and Gram-Negative Aerobic and Anaerobic Microbial Recovery of Mucosally Adherent Flora in the Middle Small Intestine*

Group	(Log_{10} CFU/mg Wet Weight [Mean ± SE])					
	Aerobes			Anaerobes		
	Total	Gram (+)	Gram (−)	Total	Gram (+)	Gram (−)
Control (n = 5)	4.4 ± 0.4	4.5 ± 0.4	3.6 ± 0.2	5.0 ± 0.3	4.5 ± 0.4	4.2 ± 0.4
Untreated (n = 5)	6.5 ± 0.1†	6.3 ± 0.1	5.9 ± 0.2‡	7.3 ± 0.3‡	7.1 ± 0.3‡	6.2 ± 0.1
Insulin (n = 5)	4.5 ± 0.5	4.4 ± 0.5	3.9 ± 0.2	4.2 ± 0.5	4.0 ± 0.5	3.5 ± 0.6
Pancreas transplant (n = 4)	4.2 ± 0.0	4.1 ± 0.1	3.5 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.1 ± 0.0

*Untreated diabetes resulted in aerobic and anaerobic microbial overgrowth, whereas insulin replacement therapy normalized microbial overgrowth.
†$p < 0.05$ versus transplant group by ANOVA and Scheffe's test.
‡$p < 0.05$ versus control, insulin, and transplant groups by ANOVA and Scheffe's test.

TABLE III
Total Gram-Positive and Gram-Negative Aerobic and Anaerobic Microbial Recovery of Mucosally Adherent Flora in the Distal Small Intestine*

Group	(Log_{10} CFU/mg Wet Weight [Mean ± SE])					
	Aerobes			Anaerobes		
	Total	Gram (+)	Gram (−)	Total	Gram (+)	Gram (−)
Control (n = 5)	5.2 ± 0.1	5.1 ± 0.1	5.0 ± 0.2	5.1 ± 0.2	5.0 ± 0.2	5.0 ± 0.0
Untreated (n = 5)	6.7 ± 0.0†	6.5 ± 0.0†	5.7 ± 0.2‡	6.9 ± 0.1†	6.7 ± 0.1†	6.1 ± 0.2‡
Insulin (n = 5)	4.6 ± 0.3	4.5 ± 0.3	4.0 ± 0.4	4.8 ± 0.3	4.7 ± 0.4	4.1 ± 0.5
Pancreas transplant (n = 4)	4.7 ± 0.2	4.7 ± 0.2	3.7 ± 0.3	4.7 ± 0.2	4.5 ± 0.2	4.2 ± 0.2

*Untreated diabetes resulted in aerobic and anaerobic microbial overgrowth, whereas insulin replacement therapy normalized microbial overgrowth.
†$p < 0.05$ versus control, insulin, and transplant groups by ANOVA and Scheffe's test.
‡$p < 0.05$ versus insulin and transplant groups by ANOVA and Scheffe's test.

Technique of pancreas donor and recipient operations: After the animal was anesthetized with 3.6% chloral hydrate, the pancreas, spleen, and duodenum were isolated. The arterial supply to the graft was preserved by isolating the aorta below the diaphragm and above the renal arteries. The graft was flushed with 0.3 mL of ice-cold normal saline. Following ligation and division of the proximal aorta and division of the distal aorta, the portal vein was divided at the liver hilum. The graft was removed and placed in cold saline. After being anesthetized with chloral hydrate and ether, diabetic recipients underwent heterotopic abdominal pancreatic transplantation. End-to-side aorto-aortic and portocaval anastomoses were performed. The spleen and the duodenum were removed after removal of the vascular clamps. All animals included in the study became normoglycemic within 24 hours of the pancreas transplantation.

Preparation of mucosal samples and culture technique: After 1 month, rats were killed, and a 5.0-mm tissue ring was removed from the proximal, middle, and distal portions of the small intestine and weighed. The tissue rings were placed in separate gassed-out transport vials containing 0.5 mL of Wilkins-Chalgren broth. Specimens were also obtained and processed for scanning elec-

TABLE IV
Predominant Microbial Recovery in the Proximal Jejunum (Log_{10} CFU/mg Tissue)

Control	*Streptococcus* species	4.5
	Lactobacillus species	3.0
	Peptostreptococcus species	3.9
Diabetic untreated	*Streptococcus* species	3.5
	Escherichia coli	5.9
	Proteus species	4.8
	Peptostreptococcus species	5.9
	Bacteroides fragilis (group)	6.1

tron microscopy. Samples were rinsed in phosphate-buffered saline to remove nonadherent bacteria and processed for aerobic and anaerobic culturing. Aerobic and anaerobic microbial recovery was expressed as the log_{10} colony-forming units (cfu)/mg tissue wet weight.

The aerobic plates were inspected at 24 and 48 hours, and all isolates were characterized by standard methods [2]. The anaerobe plates were visually inspected at 48 and 96 hours, and individual isolates were initially characterized by colonial morphology and Gram reaction. After oxygen tolerance testing, obligate microbial isolates were identified by conventional methodology [3].

Scanning electron microscopy: Tissue segments were prefixed for 24 hours in 2% glutaraldehyde buffered with 0.15 mol/L sodium cacodylate (pH 7.4). The specimens were washed twice in buffer followed by postfixation for 24 hours in osmium tetroxide. After three buffered rinses, the graft material was serially dehydrated in ethanol, critical-point dried from liquid carbon dioxide, and coated with gold-palladium. The tissue segments were viewed in a Philips 500 scanning electron microscope at 25 kV and a spot size of 8 nm (Philips Electronic Instruments, Inc., Mahwah, NJ).

Statistics: The significance of differences in microbial recovery between groups was assessed using analysis of variance and Scheffe's test.

RESULTS

Rats with untreated diabetes exhibited diarrhea, i.e., an increased frequency of watery stools. There were no deaths in the untreated group during the period of this study.

Exogenous insulin only partially normalized blood glucose levels. Wide fluctuations in these levels were noted. Frequently, glucose values ranged from 94 ± 6 mg/dL to 319 ± 12 mg/dL. In contrast, 24 hours after pancreas transplantation, all recipients were normoglycemic, and strict control of glucose homeostasis was sustained throughout the study (range: 92 ± 3 mg/dL to 123 ± 4 mg/dL). At the time the animals were killed, there was no difference in blood glucose levels between control animals (90 ± 4 mg/dL) and transplant recipients (83 ± 3 mg/dL). Animals that were receiving exogenous insulin had slightly higher serum glucose levels at the time of sacrifice (114.3 ± 7.3 mg/dL) than either the control or the transplant groups.

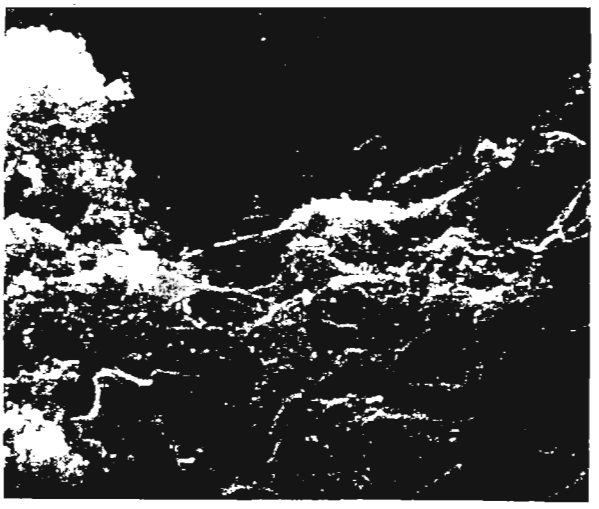

Figure 1. Scanning electron micrograph obtained from a nondiabetic control rat shows a thin mucin sheath covering the mucosal surface of the proximal small intestine with a few microorganisms embedded in this matrix (original magnification ×9,000, reduced by 40%).

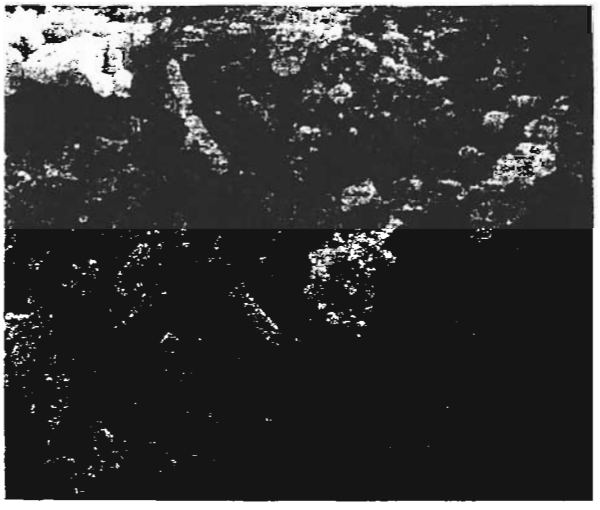

Figure 2. Scanning electron micrograph obtained from the proximal jejunum of an untreated diabetic rat shows a thick mucous sheath covering the mucosal surface in which a large heterogenous microbial population, including both rods and cocci, are embedded (original magnification ×9,000, reduced by 40%).

In rats with untreated diabetes, there was significantly increased recovery of aerobic and anaerobic bacteria in the proximal, middle, and distal small intestine as compared with age-matched nondiabetic controls (**Tables I, II,** and **III**). In the proximal intestine of the diabetic untreated rats, there were significant quantitative and qualitative increases in the recovery of facultative gram-negative organisms (*Escherichia coli*, *Proteus* species), anaerobic streptococci, and, in particular, anaerobic gram-negative rods (*Bacteroides fragilis*), compared with nondiabetic controls (**Table IV**). Similar qualitative changes also occurred in the middle and distal segments of the intestine in diabetic untreated rats.

Transplantation and exogenous daily insulin prevented the increases in aerobic and anaerobic organisms seen in the untreated diabetic rats. There were no significant differences between animals receiving insulin or an isograft compared with control rats (Tables I, II, and III).

Scanning electron micrographs obtained from nondiabetic control animals demonstrated a thin mucin sheath covering the mucosal surface of the proximal small intestine with a few microorganisms embedded in this matrix (**Figure 1**). In contrast, micrographs obtained from the proximal jejunum of untreated diabetic rats demonstrated a thick mucous sheath covering the mucosal surface in which a large heterogenous microbial population, including both rods and cocci, was embedded (**Figure 2**).

COMMENTS

Gastrointestinal symptoms such as vomiting, constipation, diarrhea, and fecal incontinence occur frequently in patients with insulin-dependent diabetes mellitus. In one survey, 76% of diabetic patients experienced one or more gastrointestinal symptoms [*4*]. The pathogenesis of gastrointestinal symptoms is believed to be multifactorial and may include disorders of intestinal motility secondary to autonomic neuropathy, bacterial overgrowth either resulting from or contributing to motility disorders, and possibly pancreatic exocrine deficiency [*5*]. In diabetes, histologic and physiologic studies support the concept of autonomic neuropathy resulting in abnormal gastrointestinal motility [*6–8*]. Disorders of intestinal motility may then result in alterations in normal intestinal peristalsis, leading to stasis and bacterial overgrowth of the proximal small intestine. With abnormal bacterial overgrowth, abnormalities of bile salt metabolism and possibly mucosal injury occur [*9*]. Overproduction of free bile acids and mucosal damage then contribute to malabsorption and diarrhea. However, conclusive studies examining small intestinal myoelectric and contractile activity in diabetic patients or animals with gastrointestinal dysfunction are lacking. Quantitative assessment of the extent of disordered intestinal motility sufficient to result in intestinal overgrowth is difficult. In one recent study, no differences in intestinal transit could be found between patients with insulin-dependent diabetes mellitus and diarrhea and normal control subjects [*10*].

Although bacterial overgrowth has been implicated in the pathogenesis of diabetic diarrhea, here, too, the data are inconclusive. Patients may still exhibit diarrhea in the absence of microbial overgrowth [*11*]. Recent clinical reports demonstrated that the presence of bacterial overgrowth in diabetics did not necessarily correlate with gastrointestinal symptoms [*12,13*]. The current study clearly demonstrates that untreated diabetes results in overgrowth of mucosally adherent microbial populations. We chose to examine the mucosally adherent population because, in contrast to other microbial populations of the gastrointestinal tract, the mucosally adherent microbial flora represent a stable ecologic population consisting of mucin-associated bacteria and mucosally adherent bacteria. The microbial-mucosal cell interaction is both metabolically and nutritionally interdependent. This population exhibits the greatest changes in response to local alterations of intestinal homeostasis in such conditions as achlorhydria, blind loop syndrome, and, as demonstrated in the current study, diabetes.

Diarrhea in the untreated diabetic rats cannot be attributed to the presence of microbial overgrowth alone. Streptozotocin does not result in complete destruction of pancreatic β cells, and, therefore, rats are not totally insulinopenic and do not develop ketosis. Rats must be allowed access to chow and water *ad libitum* for survival and, as a consequence, exhibit polyphagia and polydipsia. The resultant polyphagia must be considered in the etiology of diarrhea in the diabetic untreated rat, but, nonetheless, bacterial overgrowth could certainly exacerbate diarrhea. No significant diarrhea was noted in the treated groups. However, because of the absence of polyphagia in the rats receiving insulin replacement therapy, it is difficult to draw conclusions concerning the effect of normalization of intestinal flora on diarrhea.

A thickened mucous sheath was noted in the proximal small intestine of untreated diabetic rats compared with nondiabetic controls. This sheath contains simple and complex carbohydrates and other nutrients and serves as a nutritional source for the embedded organisms. It is seen as a direct effect of microbial overgrowth and is supportive of the quantitative recovery data. The increased recovery of gram-negative organisms in the untreated diabetic animals compared with the normal and treated animals is of interest. In general, few anaerobes are recovered from the proximal segments of the normal small bowel. There was a significantly higher mucosal recovery of facultative gram-negative organisms, anaerobic streptococci, and anaerobic gram-negative rods throughout the small intestine of the untreated animals. Similar changes in microbial populations may occur in humans with poorly controlled diabetes. For such patients undergoing surgical manipulation of the small bowel, such a finding would have implications both in terms of increased risk from infectious complications and antibiotic prophylaxis.

Support for similar changes in microbial flora in humans comes from the BB rat. This is an excellent rodent model of autoimmune-mediated diabetes mellitus with many similarities to human type I diabetes [*14*]. Preliminary studies in our laboratory found changes in microbial flora throughout the small intestine in diabetic BB rats that are identical to those seen in the current study in rats with streptozotocin-induced diabetes. Recovery of facultative gram-negative organisms, anaerobic gram-negative rods, and anaerobic streptococci was increased in rats with spontaneous diabetes compared with nondiabetic control BB rats.

In the current study, rats were entered into one of two treatment groups shortly after the induction of diabetes. Exogenous insulin, despite resultant fluctuations in blood glucose levels, was able to prevent microbial overgrowth. This suggests that sustained normalization of glucose homeostasis is not required for prevention of overgrowth. As expected, pancreatic transplantation with strict glucose control also normalized intestinal flora. Microbial metab-

olism within the gastrointestinal tract is substrate dependent. Growth within virtually all microbial populations is stimulated by glucose availability. It is likely that in untreated diabetes there is a constant overabundance of glucose. Constant unmodified increased availability of substrate would be expected to result in bacterial overgrowth and shifts in microbial populations.

The intestine represents a spatially heterogenous environment in which microbial dominance is influenced by pH, oxidation-reduction potential, species competition, and substrate. With increased availability of glucose in the small bowel for oxidative metabolism by facultative luminal and mucosally adherent bacteria, rapid oxidative metabolism would rapidly lead to a decrease in the redox potential, which favors the growth of obligate microbial populations. Therefore, a shift in microbial populations from facultative to obligate anaerobic organisms would result, as seen in the current study.

In summary, the etiology of diarrhea in untreated rats with streptozotocin-induced diabetes is multifactorial. Hyperphagia and the presence of bacterial overgrowth are factors that are likely involved. The contribution of motility disorders to both diarrhea and bacterial overgrowth is unknown. Rats with streptozotocin-induced diabetes have a reduction in the frequency of the migrating myoelectric complex that is associated with a significant increase in intestinal transit time of an intraluminally instilled bolus [*15*]. Nonetheless, critical studies examining the patterns of impaired small intestinal myoelectric and contractile activity secondary to diabetes contributing to and possibly resulting from bacterial overgrowth are lacking.

This paper is important because it addresses the curious but very important relationship between diabetes and bacterial growth. It may be as important an axis to the pancreatic transplant recipient as the amelioration of ocular and renal dysfunction and lower extremity neuropathy.

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