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Elemental Diet and IV-TPN-Induced Bacterial Translocation Is Associated with Loss of Intestinal Mucosal Barrier Function Against Bacteria

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Objective
The goal of the current study was to directly assess the role of loss of mucosal barrier function in nutritionally induced bacterial translocation.

Background
Parenteral and certain elemental enteral diets have been shown to promote bacterial translocation. The mechanisms underlying this observation, especially the question of whether nutritionally induced bacterial translocation is primarily related to loss of intestinal barrier function, *versus* an impaired immune system, remain to be fully elucidated.

Methods
Bacterial translocation was measured *in vivo*, ileal mucosal membranes were harvested, and their electrophysiologic properties and barrier function were measured *ex vivo* in the Ussing chamber system 7 days after receiving total parenteral nutrition solution parenterally (IV-TPN) or enterally (elemental diet). Chow-fed rats served as control subjects.

Results
The incidence of bacterial translocation was significantly increased both to the mesenteric lymph nodes *in vivo* and across the *in vitro* Ussing chamber-mounted ileal mucosal membranes of the elemental diet-fed and IV-TPN-fed rats. The magnitude of *Escherichia coli* and phenol red transmucosal passage in the Ussing chamber was significantly higher in the IV-TPN-fed rats than in the elemental diet-fed or chow-fed animals. The potential differences across the ileal membrane were similar between the three groups at all time points. However, the specific resistances of the ileal membranes of the IV-TPN and elemental diet groups were significantly less than the chow-fed animals, indicating increased membrane permeability.

Conclusions
Loss of intestinal barrier function plays a major role in nutritionally induced bacterial translocation, and the loss of mucosal barrier function to both *E. coli* and phenol red appeared greater in the IV-TPN than the elemental diet-fed rats.

It has been documented experimentally that parenteral and certain enteral (elemental) diets are associated with loss of intestinal barrier function, manifest as bacterial translocation,[1-6] and that protein-malnourished animals are more susceptible to lethal gut origin sepsis than normally nourished animals.[7,8] These experimental findings are consistent with randomized clinical studies documenting that patients receiving enteral feedings have a lower incidence of major infectious complications than parenterally fed patients.[9-11] Although nutritionally induced alterations in intestinal barrier function appear to be of clinical importance, many questions remain concerning the biology of this phenomenon.

To date, most studies that have examined the mucosal arm of the relationship between nutritional modulation and intestinal barrier function have not measured barrier function per se, but have largely been morphologic or biochemical studies.[1,3-5,12-16] These studies have measured nutritionally induced changes in selected parameters, such as intestinal protein, RNA or DNA content, villous height, or intestinal weight, and have assumed that a decrease in one or more of these parameters of mucosal atrophy would correlate with impaired intestinal barrier function. Equating mucosal atrophy with impaired barrier function may be too simplistic because studies in animals subjected to prolonged protein malnutrition indicate that there is no direct correlation between the development of intestinal atrophy and loss of barrier function manifest as bacterial translocation.[7,8] Similarly, reversal of intravenous total parenteral nutrition (IV-TPN)- or elemental diet-induced bacterial translocation by the oral administration of fiber[2] or the use of trophic gut hormones[17] was not associated consistently with the reversal of diet-induced alterations in intestinal morphology, weight, or protein content. The elucidation of the mechanisms responsible for diet-induced bacterial translocation is further confounded by the immunosuppressive effects of parenteral nutrition[18] and certain elemental diets.[6,19] Thus, clarification of the potential role of altered intestinal barrier function in the pathogenesis of diet-induced bacterial translocation will require functional studies of the intestinal barrier independent of other variables.

To that end and because of the inherent limitations of *in vivo* models, we have used the *in vitro* Ussing chamber model system[20] to examine the barrier function of intestinal mucosa harvested from animals subjected to various nutritional regimens. The Ussing chamber model system has the advantage that specific functions of harvested intestinal mucosal specimens can be tested directly.[21-25] Using this model system, we have been able to document that intestinal permeability to bacterial and nonbacterial permeability probes is increased in rats receiving IV-TPN or an oral elemental diet.

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MATERIAL AND METHODS

Animals

Male specific pathogen-free (SPF) Sprague-Dawley rats weighing 275 to 350 g were purchased from Harlan Breeding Laboratories (Pratsville, AL). The rats were housed in suspended wire-grid cages to limit coprophagy and eliminate the need for bedding. The rats were allowed to recover for at least 5 days after arrival before use in the experiments. The animals were maintained in accordance with the recommendations of the NIH Guide for the Care and Use of Laboratory Animals, and the experiments were approved by the Louisiana State University Medical Center, Shreveport, Animal Care Committee.

Feeding Protocol

The control group received water and rodent chow *ad libitum*. Because previous studies with rats of the same strain, approximate age, and weight had shown that the average *ad libitum* intake was 307 kcal/kg body weight/day,[2] the rats fed the elemental diet received 307 kcal/kg/day of sterile TPN solution that was placed in sterile, calibrated feeding bottles with fitted spouts. The TPN solution contained a final concentration of 4.25% amino acids (Travenol Laboratories Inc., Deerfield, IL) and 28% glucose, in addition to electrolytes and vitamins.[2] The TPN solution contained 1333 Kcal/L and the nonprotein calorie:nitrogen ration was 151:1 Kcal/g N. The IV-TPN groups had jugular venous catheters placed for the administration of the TPN solution, as described previously[2] and received between 63 and 81 mL of the TPN solution (307 Kcal/Kg) daily. All animals had continuous access to water.

Testing for Bacterial Translocation

Rats were anesthetized with intraperitoneal sodium pentobarbital (Butler Comp., Columbus, OH). The abdomen was soaked with 70% alcohol, then the skin and peritoneum were opened with sterile scissors. The exposed viscera were swabbed with a sterile, cotton-tipped applicator stick that was placed in a tube containing 5.9 mL of brain-heart infusion to detect bacterial contamination. The tip of the IV-catheter was cultured the same way. To determine translocation to the mesenteric lymph nodes (MLNs), the MLNs were excised, weighed,

and placed in 0.5 mL of brain-heart infusion, then homogenized with grinders (Tri-R Instruments, Rockville Center, NY). Aliquots of the homogenate (0.2-mL) were plated on blood agar and MacConkey's agar. The plates were examined after 24 and 48 hours of incubation at 37 C. After the MLNs had been removed for the culturing of translocating bacteria, the cecum was removed, weighed, and homogenized in brain-heart infusion. Serial dilutions of the cecal homogenate were plated onto blood agar and MacConkey agar plates, and the plates were examined after 24 and 48 hours of incubation at 37 C. Animals with positive cultures from either the catheter tip or peritoneal cavity were excluded from analysis.

Bacteria

The bacterial probe used in this study was *Escherichia coli.* This organism was chosen for several reasons: 1) it is not a pathogen and therefore, reflects the indigenous intestinal flora; 2) it is a member of the *Enterobacteriaecea,* family and gram-negative enteric bacteria are the most common organisms recovered from the organs in experimental studies of bacterial translocation[26] and from the blood of immunocomprised patients with primary bacteremias[12]; and 3) it is streptomycin-resistant, thus, allowing increased technical flexibility. The *E. coli* were grown overnight in brain-heart infusion media, washed, and resuspended in sterile phosphate buffered saline at a concentration of approximately 10^{10} colony-forming units (CFU)/mL. The initial concentration was determined spectrophotometrically and verified by pour-plate assay. The organism was cultured on MacConkey's agar plates containing streptomycin.

Ussing Chamber System

The ileal membrane was prepared essentially as described by Smith et al.[22] Briefly, a 5-cm Peyers patch-free segment of the distal ileum was harvested rapidly, opened along the mesenteric border, and washed (serially 3×) in cool medium to remove the luminal contents. Using a dissecting microscope, the serosa and the external longitudinal muscle layer were removed carefully. The ileal mucosal membrane, consisting of the mucosa and some circular muscular fibers, was then mounted in a sterilized Ussing chamber (1.12-cm^2 opening) equipped with two calomel voltage-sensitive electrodes and two Ag-AgCl current passing electrodes (World Precision Instrument Inc., Sarasota, FL). The electrodes were connected to the chamber solutions via agar bridges. Both the mucosal and serosal sides of the chamber were connected to sterilized circulating reservoirs containing Dulbeccos modified eagles medium with 20 mM glutamine (Sigma Chemical Co., St Louis, MO). The mucosal reservoir was filled with Dulbeccos modified eagles medium containing phenol red, whereas the serosal reservoir was filled with Dulbeccos modified eagles medium without phenol red. The temperature of the fluid reservoirs was maintained at 37 C by a jacketed circulating water bath, and the fluid within the reservoirs was oxygenated and driven by a gas lift column of 95% O_2/5% CO_2.

The transepithelial electrical potential difference across the mucosal membrane was measured using the calomel electrodes, and transmembrane resistance was determined using Ohm's law, by passing a 50 μA current through the membrane and measuring the change in potential difference. The resistance measurements were expressed as specific resistance (Ohms/cm^2) using the effective membrane surface area (1.12 cm^2). Potential difference is an indicator of tissue viability, whereas the specific resistance (SR) is considered to reflect tissue integrity.

The membranes were allowed to stabilize for 15 minutes. At the end of this period, a sample of medium was collected from the serosal reservoir and cultured to verify sterility of the system. In addition, this serosal fluid sample was tested for the presence of phenol red to identify otherwise unsuspected mucosal injuries (i.e., microscopic perforation). After this 15-minute stabilization period, the potential difference and specific resistance of the membrane were measured. Then, 2 mL of a washed overnight culture of *E. coli* C25 ($1-2 \times 10^{10}$ CFU/mL) was added to the mucosal reservoir, resulting in a bacterial concentration on the mucosal side of the membrane of approximately 3×10^9 bacteria/mL. Two minutes after the bacterial inoculation, a serosal fluid sample (0.5 mL) was obtained for culture as a further check of the physical integrity of the system. Preparations in which *E. coli* were present in the 2-minute serosal sample were excluded from analysis. Subsequently, serial electrical (potential difference and specific resistance) measurements were made every 30 minutes during the 180-minute experimental period. Samples (0.5 mL) of medium from the serosal compartment were collected at 60, 120, and 180 minutes and spectrophotometrically (430 nm) assayed for the presence of phenol red and quantitatively cultured. Open-circuit conditions were used in these experiments, and the pH was maintained in the range of 7.32 to 7.37.

Morphologic Structure

Intestinal mucosal samples obtained at the end of the 3-hour test period in the Ussing chamber were fixed for light microscopic examination as follows. The Dulbeccos modified eagles medium was drained from the Ussing chamber and replaced with phosphate buffered saline (PBS) containing 10% formalin. After a 30-minute incubation period, the intestinal membrane was removed

carefully from the Ussing chamber and incubated overnight in fresh 10% buffered formalin. The formalin-fixed membrane then was dehydrated to 95% ethanol and embedded in methyl methacrylate (JB-4 Polysciences Inc., Warrington, PA). Semithin (2–4 μm) sections were cut on a diamond knife and stained with 1% toluidine blue 0.

Experimental Design

The major goal of this study was to investigate the potential role of altered intestinal barrier function (permeability) in the pathogenesis of nutritionally induced bacterial translocation. Three groups of rats were studied. The first group of rats receiving water and rodent chow *ad libitum* served as the negative control group. The second group received sterile TPN solution orally (elemental diet group), and the third group received sterile TPN solution intravenously (IV-TPN group).

After 7 days, the rats were killed, their MLN complex and cecum were cultured quantitatively, and a segment of distal ileum was harvested for the *ex vivo* intestinal permeability studies. The MLNs were harvested to determine the incidence and magnitude of *in vivo* diet-induced bacterial translocation, whereas the cecum was cultured quantitatively to determine the effects of the nutritional regimen on intestinal population levels. Intestinal permeability of the distal ileum was measured in the Ussing system for 180 minutes. In these permeability studies, *E. coli* C25 (limit of detection 10 CFU/mL of serosal medium) and phenol red were used as probes to assess the permeability of the intestinal mucosa. The potential difference across the ileal membrane was measured periodically during the 180-minute experimental period to monitor the viability of the ileal mucosal membrane.

Statistical Analysis

Differences in the frequency of bacterial translocation were evaluated by the Fisher exact test. Continuous data were analyzed by a one-way analysis of variance with the post hoc Neuman-Keuls test. Bacterial counts were log-transformed before statistical analysis. Probabilities less than 0.05 were considered significant. All data were expressed as mean ± SEM, unless otherwise specified.

RESULTS

The rats fed the elemental diet gained slightly less weight than the chow-fed rats (mean weight gain 4.5 ± 1.2% *vs.* 5.7 ± 1.4%) during the 7-day experimental period, whereas the IV-TPN rats lost weight (6.4 ± 1.0% weight loss; $p < 0.05$ *vs.* chow and elemental diet groups). As previously reported,[2,17,27] intestinal bacterial overgrowth (Fig. 1) and bacterial translocation to the MLNs occurred in the rats receiving the elemental diet or IV-TPN (Table 1).

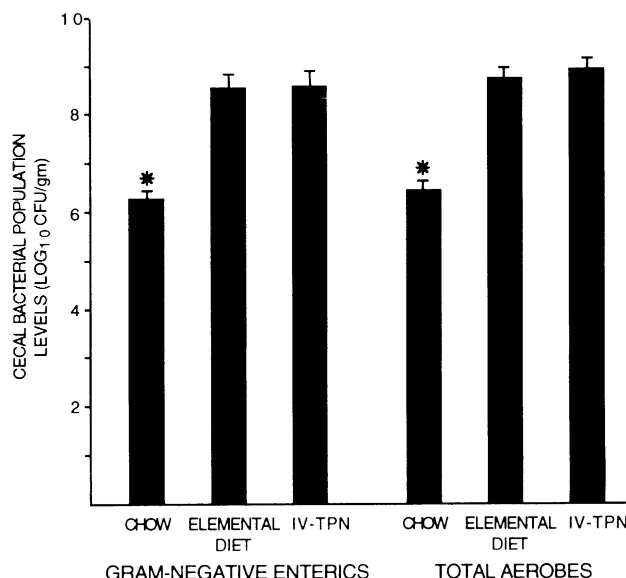

Figure 1. Intestinal bacterial population levels (mean ± SEM) are increased in rats receiving an elemental diet or IV-TPN. * $p < 0.0001$ *versus* elemental diet and IV-TPN groups.

The incidence of bacterial translocation across the mucosal membrane of the elemental diet-fed and IV-TPN-fed rats was significantly increased when tested in the Ussing chamber system, indicating that intestinal barrier function was impaired (Table 1). The ability of bacteria to cross the mucosal barrier in rats receiving the elemental diet or IV-TPN was verified histologically (Fig. 2). The time course of bacterial translocation across the ileal membranes of the rats receiving the elemental diet or IV-TPN is shown in Figure 3. Bacterial translocation had occurred by 60 minutes in 3 of the 12 rats receiving IV-TPN, but was not documented until 120 minutes for the rats receiving the elemental diet. Although *E. coli* C25 were recovered from the serosal reservoir of the IV-TPN-fed rats more often than from the elemental diet-fed rats and bacterial translocation occurred earlier in the IV-TPN-fed group, these differences did not reach statistical significance.

The magnitude of bacterial translocation *in vivo* to the MLNs and *in vitro* across the ileal membranes was similar between the IV-TPN-fed and the elemental diet-fed rats (Table 2). In contrast, the frequency (Table 1) and magnitude of the increase in ileal membrane permeability to phenol red was significantly greater in the IV-TPN-fed rats than in the elemental diet or chow-fed rats, even when the data were adjusted to look only at those membranes in which phenol red crossed (Fig. 4).

The potential difference across the ileal membranes in all three groups dropped during the first 30 minutes as

Table 1. INCIDENCE OF BACTERIAL TRANSLOCATION (BT) TO MLN AND PASSAGE OF *E. COLI* C25 AND PHENOL RED ACROSS THE ILEAL MEMBRANE *IN VITRO*

			Passage Across Ileal Membrane		
Groups	n	BT to MLN	n	*E. coli*	Phenol Red
Chow-fed	9	0%	9	0%	22%
Elemental Diet	9	89%*	17	53%†	29%
IV-TPN	12	92%*	12	75%*	75%‡

Data expressed as % of MLNs or serosal fluid specimens containing bacteria or phenol red.
* $p < 0.001$ *vs.* chow-fed.
† $p < 0.05$ *vs.* chow-fed.
‡ $p < 0.03$ *vs.* elemental diet or chow-fed.

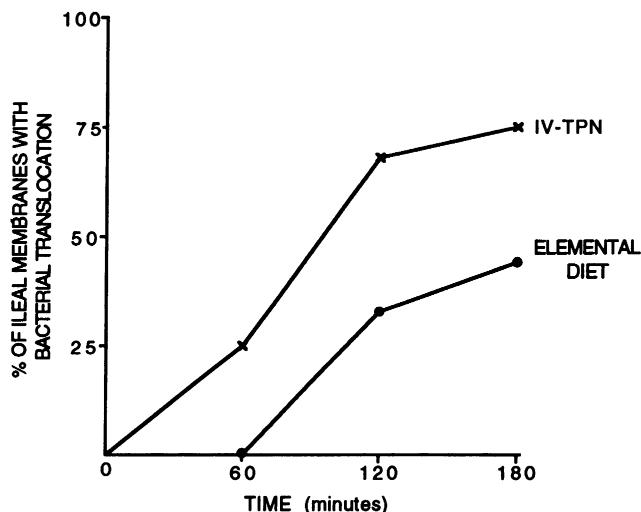

Figure 3. Time course of bacterial translocation across the ileal mucosa in rats receiving an elemental diet or IV-TPN.

the membranes equilibrated with the bathing fluids (Fig. 5). Thereafter, the potential difference remained essentially constant and was similar between the groups. In contrast, the specific resistance of the ileal membranes from animals receiving the elemental diet or IV-TPN was significantly less than that of the chow-fed rats (Fig. 6). The specific resistances did not differ at any time point between the elemental diet-fed and the IV-TPN-fed rats.

DISCUSSION

In 1949, Ussing published a now classic article evaluating active ion transport across isolated frog skin.[20] In this article, he described an apparatus composed of two individually perfused chambers that were separated from each other by frog skin. The solutions in each chamber were kept circulating by a gas mixture entering through a separate side tube. Ports were available on both sides of the membrane for sample collection or the addition of various reagents or test substances. Since then, the Ussing chamber model system has become a standard *in vitro* system for studying the physiology of electrolyte, macromolecular, and protein transport across the small intestinal or colonic mucosa and in evaluating selected aspects of gastric physiology. Most recently, Smith et al.[22] used the Ussing system to investigate the neonatal intestinal mucosal barrier and found that the transmucosal passage of bacteria was greater across the intestinal mucosa of newborn than weanling piglets. Based on this body of work, we believed that studies of harvested intestinal membranes mounted in the Ussing system might provide insight into the mechanisms underlying the process of nutritionally induced bacterial translocation.

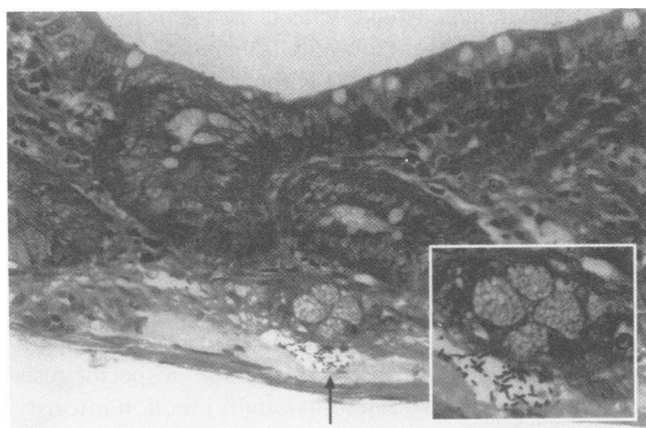

Figure 2. Histologic section of ileal mucosa stripped of seromuscular layer from a rat receiving the elemental diet showing an intact epithelial mucosa and numerous bacteria within a submucosal lymphatic × 400 (insert × 1000 magnification).

Parenteral alimentation and certain elemental diets have been documented to promote bacterial translocation, but whether bacterial translocation is related to nutritionally induced alterations in mechanical barrier function, increased intestinal bacterial population levels, impaired host antibacterial function, or a combination of these factors is unclear. Although it generally has been assumed that alteration in intestinal barrier function is a key element in nutrition-induced bacterial translocation, because of the technical limitations of current *in vivo* model systems, it has not been possible to directly test this hypothesis. Consequently, we adapted the *in vitro* Ussing chamber model system to directly measure intestinal barrier function using *E. coli* and phenol red as permeability probes in rats receiving IV-TPN or an elemental diet. The use of phenol red (molecular weight

Table 2. MAGNITUDE OF BACTERIAL TRANSLOCATION (BT) TO MLN OR PASSAGE OF *E. COLI* C25 ACROSS THE ILEAL MEMBRANE *IN VITRO*

	MLN		Ussing		
Group	Total Aerobes	Gram-Negative Enterics	All Membranes	n	Positive Membranes
Elemental diet	$1.6 \pm .8 \times 10^3$	$1.3 \pm .7 \times 10^3$	$9.6 \pm 5.3 \times 10^3$	4	$2.2 \pm .8 \times 10^4$
IV-TPN	$2.0 \pm .7 \times 10^3$	$1.5 \pm .5 \times 10^3$	$19.9 \pm 6.0 \times 10^{3}$*	9	$2.7 \pm .6 \times 10^4$

Data expressed as mean ± SEM CFU/g of MLN or CFU/serosal reservoir (n = 9–12, unless otherwise specified).
* $p < 0.01$.

376 D and molecular radius 0.70 nm) as a permeability marker has several advantages. Not only is it a pH indicator, but phenol red is not metabolized by intestinal epithelial cells, is not actively transported across the cell membrane, and has been found to be an accurate indicator of increased intestinal permeability *in vivo*.[28,29]

The results of this study documenting that the frequency and magnitude of the transmucosal passage of *E. coli* is greater in rats receiving IV-TPN or an elemental diet than in chow-fed animals provide direct evidence that intestinal antibacterial barrier function is impaired in these animals. Additionally, it appears that intestinal permeability to nonbacterial substances and bacteria is increased, because the transmucosal passage of phenol red also was increased in the IV-TPN-treated animals. These current observations are consistent with previous studies documenting sporadic areas of submucosal edema in rats receiving these test diets[2,17] and more recent studies documenting that *in vivo* intestinal permeability to horseradish peroxidase is increased in rats receiving IV-TPN or an elemental diet.[30]

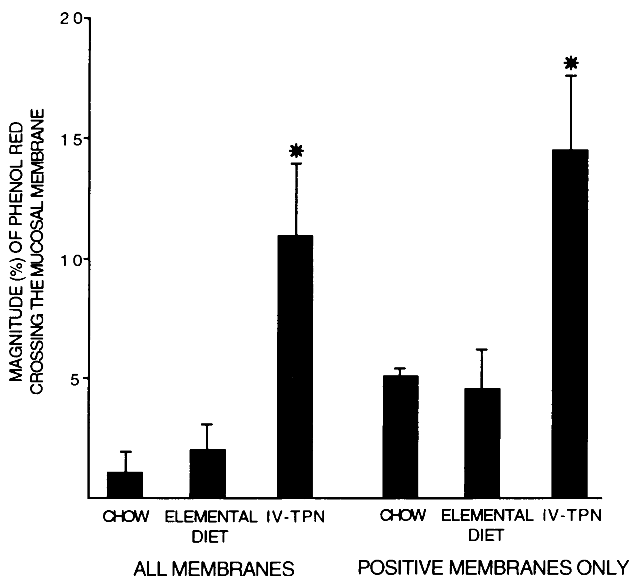

Figure 4. The magnitude of phenol red, expressed as the mean ± SEM percent of phenol red crossing the ileal membrane, was significantly higher in the IV-TPN-fed rats than in the chow-fed or elemental diet-fed rats ($p < 0.05$).

To further evaluate the effects of these test diets on intestinal function, electrophysiologic characteristics of the ileal intestinal membranes were measured. These included the potential difference, which is a marker of mucosal active ion transport, and specific resistance, which is an index of membrane integrity. Technically, membrane preparation and electrophysiologic monitoring appeared valid because the baseline electrophysiologic values of the chow-fed animals were consistent with previously published values of rat intestine.[31,32] Because the potential difference across the ileal membrane was similar between the chow and diet-fed rats, it appears that neither the administration of IV-TPN or the elemental diet diminished active ion transport of the ileal enterocytes. Consequently, it seems reasonable to conclude that the physiologic effects on intestinal barrier function associated with the administration of the elemental diet or IV-TPN were not mediated by the Na^{2+} transport system of the mucosal membranes.[33] In contrast to the potential difference, which reflects active ion transport across the mucosal membrane, transmembrane specific resistance reflects the ability of the membrane to restrict the passive movement of ions.[33] The specific resistance of membranes from the diet-treated rats was significantly less than that of the chow-fed controls at each time point, but the specific resistance did not differ at any time point between rats fed the oral and IV diets. Because a lower specific resistance corresponds to increased transmembrane nonselective ionic pathways, and the majority of passive ion flux in the intestine occurs through the paracellular pathway, the observed decrease in specific resistance is consistent with a loss in tight junction integrity. However, as reflected by the results of this study, caution must be used in directly correlating specific resistance measurements with estimates of overall intestinal permeability—i.e., although the specific resistance of the el-

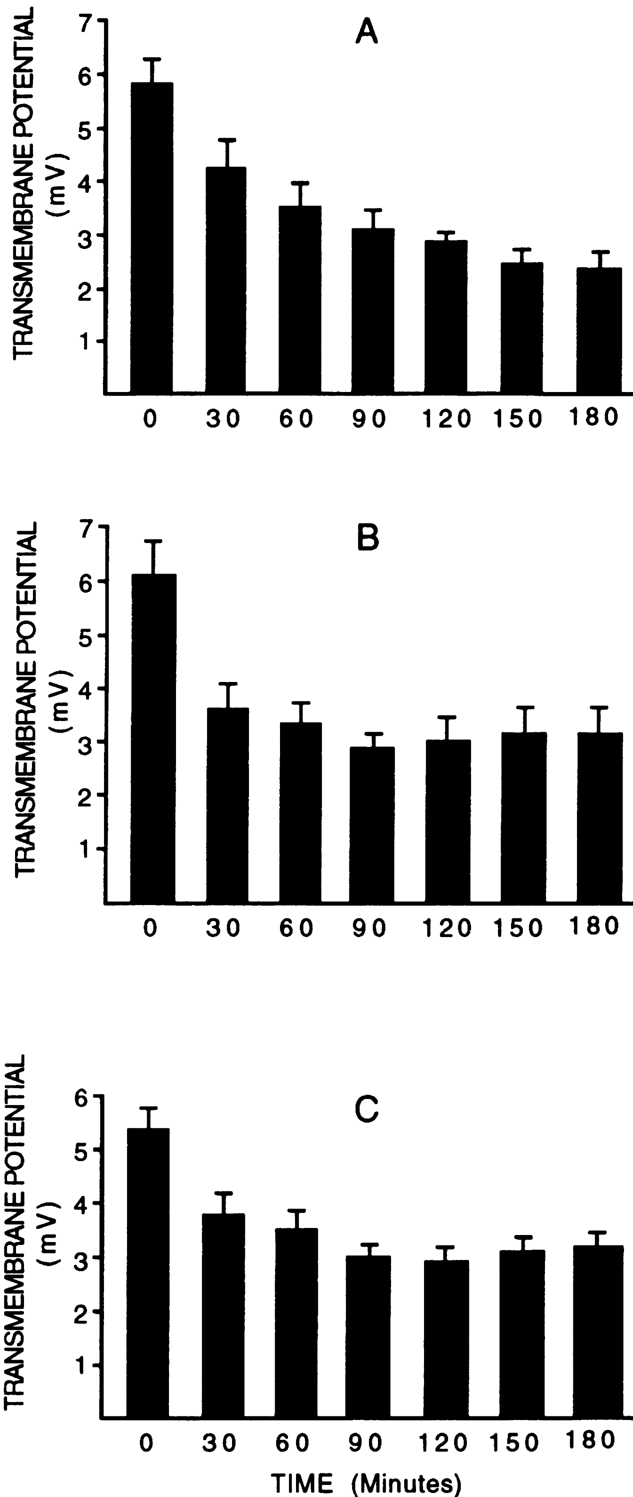

Figure 5. The transmembrane potential difference (mean ± SEM) across the ileal membranes were similar between the chow-fed (A), elemental diet-fed (B), and IV-TPN-fed (C) rats.

emental diet and IV-TPN-fed rats were equally decreased, the frequency and magnitude of the transmembrane passage of phenol red was significantly greater in the IV-TPN-fed than the elemental diet-fed animals. One explanation for these findings is that the upper limit of the size of the transmembrane or paracellular pathways was increased more in the IV-TPN-fed than in the elemental diet-fed animals.

Because we were not able to directly visualize *E. coli* in the process of crossing the mucosal membrane, we cannot determine the relative role of the transmembrane *versus* the paracellular pathway of bacterial translocation. However, studies with polarized epithelial cell cultures[34,35] indicate that bacteria translocate across enterocytes in a transcellular fashion as do the *in vivo* studies

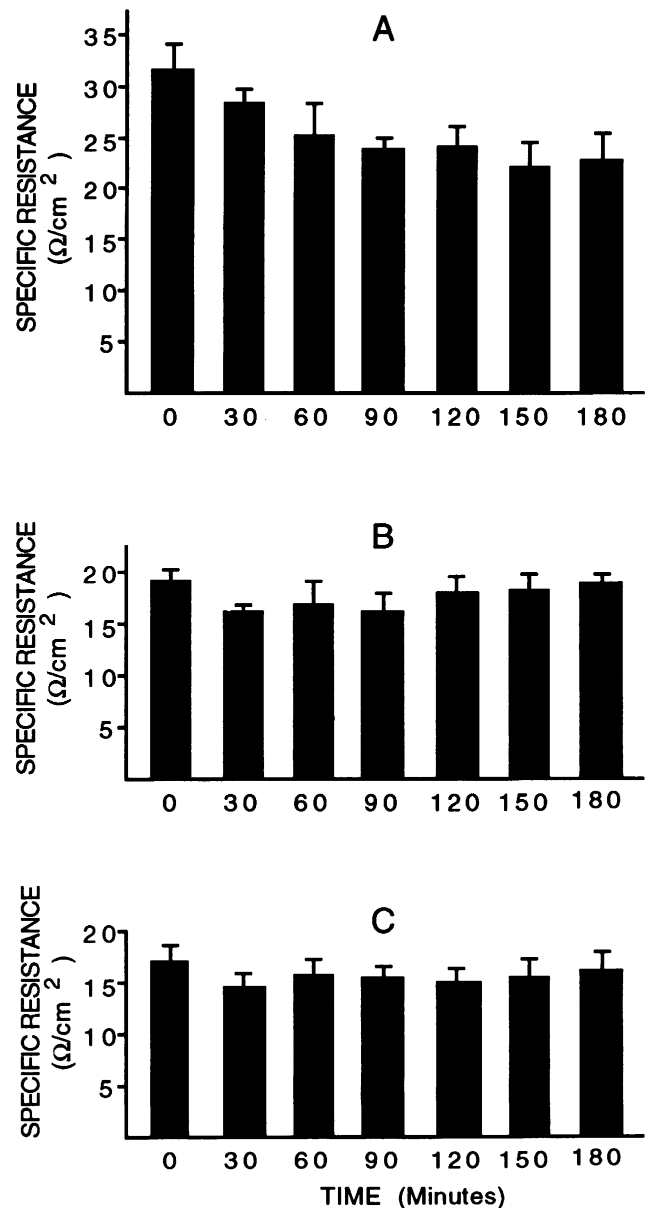

Figure 6. The specific resistance (mean ± SEM) of the chow-fed rats (A) was significantly increased compared with the elemental diet-fed (B) or IV-TPN-fed (C) rats ($p < 0.01$ at times 0, 30 and 90 minutes; $p < 0.05$ at 120, 150 and 180 minutes IV-TPN, and $p < 0.05$ at 120 minutes elemental diet).

of Alexander et al.[36] and Wells et al.[37] Furthermore, in models of bacterial translocation where the mucosal barrier is intact, there is no evidence documenting that bacterial translocation occurs in a paracellular fashion. These results, in combination with the fact that the frequency of transmucosal *E. coli* but not phenol red passage was increased in the elemental diet-fed rats, adds indirect support to the concept that bacterial translocation occurs transcellularly. On the other hand, our ability to histologically identify *E. coli* in submucosal lymphatics is consistent with previous *in vivo* studies, indicating that the primary route by which translocating bacteria reach systemic tissues after they have crossed the mucosal barrier is via the lymphatics.[26]

The observation that the loss of mucosal barrier function to both *E. coli* and phenol red appeared greater in the IV-TPN than the elemental diet-fed rats is of potential clinical interest, especially when taken together with the large body of experimental data documenting that host immune function is better preserved with enteral *versus* parenteral feeding.[38] These results also are consistent with randomized clinical studies documenting that trauma and burn patients receiving enteral feedings have a lower incidence of major infectious complications than parenterally fed patients.[9,10,11] The current results also help explain the findings of most clinical trials of TPN, which have revealed that the administration of TPN increases the incidence of infectious complications in all but the most severely malnourished patients.[39-41]

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