Persistent epithelial dysfunction and bacterial translocation after resolution of intestinal inflammation

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The intestinal epithelium plays an essential role in host defense by preventing the entry of bacteria and other potentially harmful substances into the lamina propria. In addition to diluting luminal toxins, the secretion of water and mucus by the epithelium may assist in preventing the adherence and invasion of microbes. Impairment of epithelial barrier function and/or secretion could therefore result in an increased susceptibility to infection and inflammation. Inflammation of the gastrointestinal mucosa in various species is associated with the release of a number of soluble mediators that have the capacity to influence intestinal epithelial function (49, 53, 54). For example, interleukin (IL)-4 and prostaglandin D2 have been shown to decrease epithelial chloride secretion, and IL-4, IL-13, and interferon-γ have been shown to diminish epithelial barrier function (9, 25, 30, 56). It has previously been demonstrated, in various species, that inflammation of the colon is accompanied by an acute reduction in epithelial responsiveness to secretagogues (3, 15, 22, 29). We have further shown that, in the rat, such changes are long lasting, at least when assessed in vitro. Thus the colonic epithelium was found to be hyporesponsive to electrical field stimulation, IBMX, and carbachol for up to 12 wk after induction of colitis (1). Indeed, we have recently observed that the hyporesponsiveness to electrical field stimulation is still apparent in rats 27 wk after a bout of colitis (unpublished observations). This epithelial dysfunction was attributed, at least in part, to the release of nitric oxide (NO) from the inducible isoform of NO synthase (iNOS) (1). Altered colonic secretion following a bout of inflammation could be attributable to a number of factors, including changes in the enteroecto itself or to the other cells that participate in the secretory response to various agonists (e.g., nerves, mast cells). For example, since enteric nerves play an important role in the secretory response to some agonists (7, 51), it is possible that alterations in innervation of the colon or in neurotransmitter release would result in epithelial hyposecretion. Indeed, it is noteworthy that significant decreases in the release of norepinephrine from myenteric nerves have been observed in rats following a bout of colitis (20). Interestingly, these changes were observed in parts of the intestine that were remote to the site of inflammation (20). Diminished expression of...
binding sites for substance P has been documented in experimental colitis (12) and could contribute to the hyposecretion to agonists that induce secretion through the release of this neuropeptide. In the previous in vitro studies, we observed that the colonic secretory response was substantially diminished with three distinct agonists, suggesting that changes at the level of the enterocyte most likely accounted for the observed hyporesponsiveness (1).

In the present study, we have investigated the possibility that colonic epithelial secretory dysfunction occurs in vivo and that it persists for many weeks after resolution of a bout of colonic inflammation. We also tested the hypothesis that small intestinal inflammation could produce prolonged alterations in colonic epithelial secretion. We then examined whether the functional consequences of altered secretory function following a bout of colitis or enteritis might include an increase in bacterial colonization of the colon and bacterial translocation across the epithelium, as well as whether or not any such changes could be reversed by an inhibitor of iNOS. Finally, we examined the possibility that a bout of colitis leads to prolonged changes in epithelial proliferation, which could contribute to alterations in secretory and barrier function.

MATERIALS AND METHODS

Animals. Male rats of the Wistar, Hooded-Lister, and Sprague-Dawley strains weighing 175–200 g were obtained from Charles River Breeding Farms (Montreal, PQ, Canada). The rats were fed standard chow and water ad libitum. All experiments described below were approved by the Animal Care Committee of the University of Calgary and were performed in accordance with the guidelines of the Canadian Council on Animal Care.

Induction of colonic inflammation. Colitis was induced in Wistar rats as previously described (1, 35). Briefly, a plastic catheter inserted rectally into the colon ~8 cm proximal to the anus was used to instill 30 mg of 2,4,6-trinitrobenzene sulfonic acid (TNBS) dissolved in 0.5 ml of 50% ethanol (vol/vol). A group of age-matched rats, which were left untreated during the course of the study, served as controls. In all experiments, the minimum sample size per group was five.

Colonic fluid secretion was measured 6 wk after TNBS administration and in controls. In separate groups of rats, permeability of the gastrointestinal tract to $^{51}$Cr-EDTA was assessed (72 h and 6 wk after intracolonic administration of TNBS or of the 50% ethanol vehicle) and in age-matched, untreated rats, after which samples of colonic tissue were fixed in neutral buffered formalin for histological evaluation. The measurement of permeability to $^{51}$Cr-EDTA involved placing rats in individual metabolic cages and collecting urine over a period of 12 h. The rats (n = 6 per group) were given 100 $\mu$Ci of $^{51}$Cr-EDTA (33 Ci/mmol) orally, after which the collection of urine was initiated. The urine samples were then counted in a gamma spectrometer, and the fractional excretion of the $^{51}$Cr-EDTA was calculated. The appearance of $^{51}$Cr-EDTA in the urine is a measure of total gastrointestinal tract permeability. To determine the extent to which colonic inflammation had resolved 6 wk after intracolonic administration of TNBS or the 50% ethanol vehicle, colonic tissue was excised for measurement of myeloperoxidase activity (MPO) and prostaglandin E$_2$ synthesis (3).

Induction of small intestinal inflammation. Sprague-Dawley rats were infected with ~3,000 stage-III Nippostrongylus brasiliensis larvae, as previously described (2). A group of age-matched rats served as controls. In this model, infection of the small intestine (primarily jejunum) occurs 60–120 h after the administration of the larvae, and the infection and the associated small intestinal inflammation is typically cleared by 18–21 days after infection (18, 36, 37). Tissue for histological analysis was taken from the small intestine and colon 52 days after administration of the larvae. A separate group of rats was used for measurement of colonic fluid secretion (see In vivo colonic fluid secretion).

Samples of the small intestine and colon of naive rats and rats infected with N. brasiliensis 52 days earlier were fixed in Carnoy’s solution and processed using routine histological techniques. Sections (7 µm) were mounted on glass slides and stained with hematoxylin and eosin. Coded sections were then assessed for degree of inflammation by an observer who was unaware of the treatments the rats had received.

In vivo colonic fluid secretion. Rats were fasted for 24 h and anesthetized with 20% urethane (6 ml/kg), and a laparotomy was performed. Colonic loops (~5 cm) were formed using suture to tie off each end of the distal colon (i.e., the area of previous exposure to TNBS). Care was taken not to disrupt the blood and nerve supplies to the gut. A cannula was inserted into one of the ends of each loop. Clostridium difficile-derived toxin A (10 µg) dissolved in 2 ml of Tyrode’s buffer (in mM: 133 NaCl, 4.7 KCl, 1.84 MgSO$_4$, and 20 NaHCO$_3$; pH 7.4) was injected into the colonic loops. In control rats, loops were injected with Tyrode’s buffer alone. In other experiments, 2 ml of Tyrode’s buffer containing the phosphodiesterase inhibitor IBMX (300 µM) or vehicle were injected instead of toxin A. IBMX was used as another agonist because we found that the epithelium of previously inflamed colon is hyporesponsive to this secretagogue in vitro (1). After 3 h, the loops were carefully excised and the volume of fluid recovered from the loops was recorded. Any changes in the volume of fluid during this period would reflect the difference between secretion and absorption (6, 33, 34). Thus, in the present study, we have used the volume of fluid recovered from the colonic loops as an index of secretion. The dry weight of the tissue was determined after desiccation at 60°C for 24 h. Changes in fluid accumulation after administration of C. difficile toxin A could also be attributable to epithelial damage caused by this agent. To test this hypothesis, we used leakage of lactate dehydrogenase into the lumen as an index of cellular disruption. A spectrophotometric assay for lactate dehydrogenase was performed on samples of the luminal fluid from vehicle- and toxin A-treated rats according to a previously described protocol (5).

Role of iNOS. To determine the contribution of iNOS activity to any changes in colonic fluid secretion induced by a prior bout of colitis, rats were treated with 10$^4$-iminooethyl-l-lysine (l-NIL; 3 mg/kg ip) or vehicle (saline) 10 min before measurement of colonic fluid secretion. This dose of l-NIL was chosen because it has previously been shown to reverse an endotoxin-induced decrease in blood pressure but had no effect on the blood pressure of normal rats (44). Effects of TTX. To determine the role of nerves in the hyporesponsiveness observed in rats 6 wk after a bout of colitis, experiments were performed as described in In vivo colonic fluid secretion in which IBMX was used as a secretagogue, but in one-half the rats, (n = 6/group) TTX (10 µM)
was added to the fluid that was injected into the colonic loop. This concentration of TTX exceeds that previously shown to significantly inhibit colonic secretion induced by C. difficile toxin A (7).

**Mucus secretion.** Rats (n = 5–9 per group) that had been fasted for 24 h were given [3H]glucosamine (20 μCi) intraperitoneally to quantitate mucus secretion (4). To examine basal mucus secretion, the rats were killed 3 h after injection of the labeled glucosamine. The colon was excised, and the fecal contents were removed before gentle scraping of the luminal surface with a glass slide. These samples were subsequently processed for quantification of the labeled glycoproteins (see below). To examine stimulated mucus secretion, rats were anesthetized with pentobarbital sodium (65 mg/kg ip) 3 h after injection of the labeled glucosamine. Colonic loops (~2 cm long) were formed using suture to tie off each end, and a cannula was inserted into one end of the loop. Previous studies have established that the creation of the loop per se does not induce mucus secretion or cause goblet cell depletion (8). Carbocoll (300 μl of a 10 μM solution) was instilled into mucosal loops and left in place for 30 min. At the end of this period, the luminal surface of the loop was gently scraped with a glass slide, as described above. The samples collected in both sets of experiments (basal and carbocoll-stimulated) were placed in 5 ml of PBS and were then vortex mixed. After centrifugation at 1,000 g for 10 min, the supernatant was mixed with an equal volume of 10% trichloroacetic acid-1% phosphotungstic acid for 30 min at 4°C to precipitate the glycoproteins. Following centrifugation (2,000 g; 10 min), each pellet was mixed with 4 ml of scintillation cocktail (Ecolite; ICN Biomedicals, Irvine, CA) and counted in a liquid scintillation counter. The amount of radiolabeled glycoprotein secreted was then calculated as counts per milligram of protein per centimeter of colon.

**Antigen-induced colonic secretion in vitro.** In experiments examining colonic epithelial responses to antigen, Hooded-Lister rats were sensitized by intraperitoneal injection of a 1.0-ml solution containing 10 μg of ovalbumin and 1.0 mg of aluminum hydroxide adjuvant, prepared as previously described (39). Adjuvant alone was injected in control animals. Two days later, sensitized and nonsensitized control rats were divided into two groups. Colitis was induced in one group of sensitized and one group of control rats by intracolonic administration of TNBS, as described in Induction of colonic inflammation, and the remaining rats were left without any further treatment. Following a 6-wk time period to allow for the colonic inflammation to resolve, blood was taken from each rat for measurement of ovalbumin-specific reaginic antibody titers by the passive cutaneous anaphylaxis (PCA) reaction (38). Rats were considered sensitized if the serum diluted to 1:32 caused a positive PCA reaction.

The distal colon from sensitized and control rats was excised and gently washed of fecal contents. The colon was stripped of its external muscle by blunt dissection, and a segment of mucosa from each rat was mounted in an Ussing-type diffusion chamber (Navygate, Sparks, NV). Experiments were carried out as previously described in detail (1). Briefly, after a 20-min equilibration period, the short-circuit current (Isc) response to serosally applied ovalbumin (100 μg/ml) was measured. This concentration of ovalbumin was chosen because it had previously been shown to cause a maximal Isc response in the rat (39). Following the completion of these experiments, luminal fluid from the Ussing chambers was taken for measurement of rat mast cell protease (RMCP)-II concentrations. The release of this protease is a sensitive index of mucosal mast cell degranulation (45). Briefly, this entailed protein precipitation by addition of an equal volume of 10% TCA to the luminal fluid, followed by centrifugation at 12,000 g for 90 min at 4°C. Protein samples from each tissue were then run on a 12.5% SDS-polyacrylamide gel, and RMCP-II levels were measured by Western blot detection with a rabbit anti-RMCP-II antibody.

**Bacterial translocation and colonic bacterial levels.** Six weeks after administration of TNBS or vehicle (0.5 ml of 50% ethanol) or 52 days after infection with N. brasiliensis, rats were killed by cervical dislocation (appropriate controls were killed at the same time). The mesenteric lymph node complex and spleen were removed from each rat using sterile technique, as previously described (24). Each tissue was then weighed and homogenized in 5 ml of sterile PBS. Two serial 10-fold dilutions were made of each sample, and 0.1-ml aliquots of each undiluted and diluted sample were plated onto MacConkey and blood agar plates. Following incubation at 37°C for 48 h, bacterial counts were performed. Any mesenteric lymph node or splenic tissue having >20 colony forming units (CFU)/g tissue was classified as positive for translocation of bacteria.

**To examine total colon aerobic bacterial levels (luminal plus adherent and invasive), a segment of colon including luminal contents was placed in 5 ml of sterile PBS. To examine the number of bacteria adherent to or invading the colonic tissue, a second tissue sample (excluding the luminal contents) was placed in 5 ml of PBS and vortexed vigorously for 5 s (24). These samples were then transferred to tubes containing sterile PBS and vortexed again. This was repeated a total of four times. Finally, both tissue samples (for total and for adherent/invasive bacteria) were weighed, homogenized, and diluted before plating of 0.1 ml of each dilution on MacConkey and blood agar plates. Once again, the number of CFU per gram of tissue was recorded for both naïve and TNBS-treated animals. To examine total colonic anaerobic bacterial levels (luminal plus adherent and invasive), a segment of colon including luminal contents was placed in 5 ml of sterile, prereduced brain-heart infusion broth. Following homogenization, the samples were transferred into an anaerobic chamber and diluted in prereduced brain-heart infusion broth before plating of 0.1 ml of each dilution onto blood agar plates. The plates were incubated in anaerobic conditions at 37°C for 72 h, after which bacterial counts were performed as above.

To examine the induction of iNOS-derived NO to bacterial colonization and translocation after a bout of colitis, rats treated 5 wk earlier with TNBS (n = 12) were treated twice daily with L-NIL (3 mg/kg ip) for 7 days. A second group of rats (n = 12) was treated with vehicle (saline) instead of L-NIL.

**Colonic epithelial proliferation.** The detection of proliferating cells in the colonic epithelium was compared in groups of rats treated intracolonically with either TNBS/ethanol or ethanol alone or in rats left untreated. Six weeks later, the rats were given bromodeoxyuridine (BrdU; 150 mg/kg). One hour later, the rats were anesthetized with pentobarbital (65 mg/kg ip) and intravascularly perfused with 10% formalin. The colon was removed, and cryostat sections (10 μm) were mounted on chromium phosphate-gelatin coated slides. Immunohistochemistry was then performed using anti-BrdU and goat anti-donkey Cy3 secondary antibodies (Jackson Laboratories). Briefly, tissues were washed in Tris buffer containing 0.1% Triton X-100 followed by a 30-min incubation in 1 M HCl. After two washes in Tris buffer, slides were incubated with primary antibody (1:500 dilution of anti-BrdU) overnight at 4°C. Following another series of washes, slides were incubated with secondary antibody (1:1,000 dilution of donkey anti-rat) at room temperature for
Slides were examined with a fluorescence microscope and a Cy3 filter by an observer who was unaware of the treatments the rats had received. The average number of BrdU-positive cells per villus-crypt unit was determined (at least 6 randomly selected villus-crypt units were examined, and the number of BrdU-positive cells was then averaged). BrdU labeling experiments were also performed using rats that had been treated twice daily with L-NIL (as described in Bacterial translocation and colonic bacterial levels) or vehicle for 1 wk (beginning 5 wk after intracolonic administration of TNBS or the 50% ethanol vehicle; n = 6/group).

Statistical analysis. All data are expressed as means ± SE. Comparisons among groups of data were made using a one-way ANOVA followed by the Student–Newman–Keuls Test, with the exception of the bacterial translocation incidences, for which the Fisher’s Exact test was used. With all analyses, an associated probability (P value) of <5% was considered significant.

Materials. TNBS was obtained from Fluka Chimika (Buchs, Switzerland). Alcian blue SGX, IBMX, ovalbumin, and L-NIL were obtained from Sigma Chemical (Mississauga, ON, Canada). C. difficile toxin A was purchased from Techlab (Blacksburg, VA). Safranine was obtained from Fischer Scientific (Nepean, ON, Canada). The bacterial culturing materials were obtained from Becton Dickinson (Cockeysville, MD). RMCP-II and the antibody to this protease were obtained from Moredun Animal Health (Glasgow, UK). [3H]glucosamine was obtained from ICN Biomedicals (Costa Mesa, CA). All other materials were obtained from VWR (Edmonton, AB, Canada) or Sigma Chemical.

RESULTS

Six weeks after TNBS administration, the histological appearance of the colon was indistinguishable from that of naive controls, confirming our previous observations (1). Although acute colitis, induced either by TNBS/ethanol or ethanol alone, was associated with a significant increase in gastrointestinal permeability to $^{51}$Cr-EDTA, the permeability had returned to normal by 6 wk after TNBS/ethanol or ethanol administration (Fig. 1). Similarly, acute colitis was associated with significant increases in colonic MPO activity and colonic PGE$_2$ synthesis, but by 6 wk after TNBS/ethanol or ethanol administration, these changes were no longer evident (Fig. 1).

Effects of a prior bout of colitis on colonic secretion. In naive rats, toxin A from C. difficile caused approximately a fivefold increase in fluid secretion into the colonic loops over that observed in vehicle-treated controls (Fig. 2A). In rats in which colitis had been induced 6 wk earlier, the fluid accumulation in colonic loops after exposure to toxin A was significantly lower than that in vehicle-treated controls (reduced by 53%; Fig. 2A). More striking was the observation that the secretory response to IBMX was completely abolished in the rats that had previously had colitis (Fig. 2B).

Any change in fluid accumulation after exposure to C. difficile toxin A could be, at least in part, to exudation of intracellular and extracellular fluid subsequent to injury. Thus differences among the treatment groups could be attributable to differences in the severity of damage rather than to differences in secretion. To test this hypothesis, we measured levels of lactate dehydrogenase in the luminal fluid samples as an index of damage. We found that there were no significant differences in luminal levels of lactate dehydrogenase among the four groups (as a percentage of the naive group treated with vehicle: naive/toxin A: 98 ± 9%; TNBS/vehicle: 104 ± 35%; TNBS/toxin A: 74 ± 11%).

Effects of a prior bout of colitis on colonic mucus secretion. Although a prior bout of colitis resulted in markedly diminished fluid secretion, it did not significantly affect basal or carbachol-stimulated colonic mucus secretion (13.4 ± 2.0 vs. 11.8 ± 2.3 cpm·μg AJP-Gastrointest Liver Physiol • VOL 281 • SEPTEMBER 2001 • www.ajpgi.org

Statistical analysis. All data are expressed as means ± SE. Comparisons among groups of data were made using a one-way ANOVA followed by the Student–Newman–Keuls Test, with the exception of the bacterial translocation incidences, for which the Fisher’s Exact test was used. With all analyses, an associated probability (P value) of <5% was considered significant.

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Exposure to carbachol for 30 min caused an 20-fold increase in [3H]glucosamine-labeled glycoprotein release in both naïve and postcolitis rats (202.4 ± 44.3 vs. 238.4 ± 45.6 cpm·μg protein⁻¹·cm colon⁻¹, respectively). 

Effects of a prior bout of enteritis on colonic secretion. The effects of antigen (ovalbumin) on chloride secretion by the colon were examined in vitro in postcolitis rats vs. naïve controls. Addition of the antigen to the serosal side of the colonic tissue from naïve, sensitized rats resulted in a 10-fold increase in $I_{sc}$, which is generated primarily by active chloride secretion into the lumen (Fig. 3). When the same experiments were performed in naïve rats that had not been sensitized to the antigen, no secretory response was observed. Antigen challenge of colonic tissue from sensitized, postcolitis rats did not elicit a secretory response (Fig. 3). Indeed, the response to antigen was no different from that in postcolitis rats that had not been sensitized to the antigen. The absence of a secretory response to antigen in the sensitized postcolitis rats was not due to a failure of the antigen to cause mucosal mast cell degranulation, since we observed a significant increase (3-fold) in the luminal levels of the mucosal mast cell protease RMCP-II in these rats. A doubling of RMCP-II concentrations in the luminal side of the Ussing chamber was noted in naïve, sensitized rats following challenge with the antigen. Moreover, all rats used in this study as “sensitized” were confirmed to be sensitized by a positive PCA test.

Effects of a prior bout of enteritis on colonic mucus secretion. Colonic tissue that was fixed 52 days after N. brasiliensis infection did not exhibit any histological evidence of inflammation. Toxin A caused a significant increase (2.8-fold) in fluid accumulation in the colon of control rats (Fig. 4). In colonic loops from rats previously infected with N. brasiliensis, toxin A caused significantly less fluid accumulation than in controls (33% reduction). There was no significant difference in the amount of fluid recovered from toxin A-treated vs.

Fig. 2. Effects of a prior bout of colitis on colonic fluid secretion stimulated by Clostridium difficile toxin A (A; 5 μg/ml) or IBMX (B; 300 μM). Colonic fluid levels were measured 3 h after administration of toxin A, IBMX, or vehicle. Values are means ± SE, with 5–8 rats per group. *P < 0.05, **P < 0.01 vs. corresponding vehicle-treated group. δP < 0.05 vs. corresponding naïve group.

Fig. 3. Changes in colonic short-circuit current ($I_{sc}$) following challenge with ovalbumin. Tissues from rats killed 6 wk after induction of colitis and age-matched naïve controls were mounted in Ussing chambers. In each case, subgroups of rats were sensitized to ovalbumin. Exposure of the tissue from sensitized, naïve rats to the antigen resulted in a significant increase in $I_{sc}$ (**P < 0.01) that was not seen in either of the groups of nonsensitized rats. Moreover, exposure of sensitized, postcolitis rats to the antigen did not evoke a change in $I_{sc}$. δP < 0.01 vs. corresponding naïve group.

Fig. 4. Effects of a prior bout of enteritis on C. difficile toxin A-stimulated colonic fluid secretion. Colonic fluid levels were measured 3 h after stimulation with toxin A (5 μg/ml) or vehicle. Values are means ± SE, with 6–9 rats per group. **P < 0.01 vs. corresponding vehicle-treated group. δP < 0.05 vs. corresponding naïve group.
vehicle-treated loops in rats previously infected with *N. brasiliensis*.

**Effects of iNOS inhibition on colonic secretion.** Treatment with L-NIL 10 min before toxin A administration did not have any effect on colonic fluid accumulation in naïve rats (Fig. 5). Similarly, L-NIL pretreatment did not affect colonic secretion in rats treated with TNBS 6 wk earlier; that is, the volume of fluid recovered from toxin A-treated colonic loops in postcolitis rats remained significantly reduced compared with the volume recovered from controls. In contrast, in IBMX-stimulated colonic loops, the volume of fluid recovered in postcolitis rats pretreated with L-NIL was not different from the volume of fluid recovered from naïve rats (i.e., L-NIL partially reversed the hyposecretion; Fig. 5).

**Effects of TTX.** Pretreatment with TTX did not significantly affect the hyporesponsiveness to IBMX in rats 6 wk after induction of colitis (Fig. 6). TTX also had no significant effect on the secretory response to IBMX in control rats.

**Colonic bacterial levels and bacterial translocation: effects of a prior bout of colitis.** Despite the normal macroscopic and histological appearance of the colon, there were marked changes in bacterial colonization of the colon 6 wk after induction of colitis. We observed a 16-fold increase in the number of aerobic bacteria in the colon (tissue + lumen) of postcolitis rats compared with controls (Table 1). However, there was no difference in the number of aerobic bacteria between control and postcolitis rats when only tissue levels (adherent + invasive) were examined ($4.4 \pm 0.6$ vs. $5.0 \pm 0.5 \log_{10}$ CFU/g, respectively). Total gram-negative bacterial levels in the colon ($4.4 \pm 0.9$ vs. $4.2 \pm 0.9 \log_{10}$ CFU/g) and total anaerobes ($7.1 \pm 0.5$ vs. $7.4 \pm 0.5 \log_{10}$ CFU/g) were also not different between control and postcolitis rats, respectively. As shown in Table 1, aerobic bacterial translocation to either the mesenteric lymph nodes or spleen was observed in 77% of the rats examined 6 wk after administration of TNBS, significantly greater ($P < 0.0005$) than the 23% incidence in naïve rats. In terms of the types of bacterial colonization in the postcolitis rats vs. naïve controls, there were few major differences. *Escherichia coli* and *Enterococci* species were the predominant bacteria in both naïve and postcolitis rats.

![Fig. 5. Effects of a selective inducible nitric oxide synthase (iNOS) inhibitor, N²-iminoethyl-L-lysine (L-NIL; 3 mg/kg), on in vivo colonic fluid responses to toxin A (A; 5 µg/ml) and IBMX (B; 300 µM). Values are means ± SE, with 7–9 rats per group. *bP < 0.01 vs. corresponding naïve group. There were no significant differences between the corresponding L-NIL- and vehicle-treated groups.](http://ajpgi.physiology.org/)

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample Size</th>
<th>Total Aerobes, log_{10} CFU/g</th>
<th>Incidence of Translocation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>22</td>
<td>$5.3 \pm 0.5$</td>
<td>23</td>
</tr>
<tr>
<td>6 wk post ethanol</td>
<td>8</td>
<td>$5.3 \pm 0.1$</td>
<td>25</td>
</tr>
<tr>
<td>6 wk post TNBS</td>
<td>22</td>
<td>$6.5 \pm 0.3^*$</td>
<td>77†</td>
</tr>
<tr>
<td>6 wk post TNBS + vehicle</td>
<td>12</td>
<td>$6.3 \pm 0.2$</td>
<td>75</td>
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<tr>
<td>6 wk post TNBS + L-NIL</td>
<td>12</td>
<td>$6.3 \pm 0.3$</td>
<td>33#</td>
</tr>
</tbody>
</table>

Studies were performed in male Wistar rats killed 6 wk after intracolonic administration of trinitrobenzene sulfonic acid (TNBS) or the ethanol vehicle and in age-matched, naïve controls. Bacterial colonization was measured in samples of the colonic tissue plus the luminal contents. Bacterial translocation was determined as described in MATERIALS AND METHODS. In other experiments, rats that had been given TNBS intracolonically 5 wk earlier were treated twice each day for 1 wk with N⁰-iminoethyl-L-lysine (L-NIL; 3 mg·kg⁻¹·day⁻¹ ip) or vehicle. The bacterial colonization and percent translocation were then determined. *P < 0.05, †P < 0.0005 vs. vehicle or controls; #P < 0.05 vs. TNBS + vehicle.
Administration of the vehicle for TNBS (0.5 ml of 50% ethanol) did not induce significant changes in bacterial colonization of the colon or in bacterial translocation. When examined 6 wk after intracolonic administration of the vehicle, the levels of aerobic and gram-negative bacteria (5.3 ± 0.1 and 4.5 ± 0.1 log_{10} CFU/g, respectively) were not different from those in naive controls. Likewise, an incidence of 25% bacterial translocation was not different from the 23% rate of translocation in naive controls (Table 1).

In postcolitis rats treated with L-NIL for 7 days, the incidence of bacterial translocation was 33%, significantly less (P < 0.05) than the rate observed in naive rats (77%) and than the rate observed in postcolitis rats treated with the vehicle (75%). However, the total numbers of aerobic (Table 1) and anaerobic bacteria in the colons of rats treated with L-NIL were not significantly different from those in the rats treated with vehicle (7.4 ± 0.5 log_{10} CFU/g in TNBS + vehicle group vs. 7.3 ± 1.0 log_{10} CFU/g in TNBS + L-NIL group).

Colonic bacterial levels and bacterial translocation: effects of a prior bout of enteritis. In Sprague-Dawley rats that had been infected with *N. brasiliensis* 52 days earlier, the colonic aerobic bacterial numbers were 10-fold higher (P < 0.05) than in naive controls (Table 2). Unlike the case in the postcolitis rats, there was also a significant increase (25-fold) in the numbers of gram-negative bacteria in the colons of the postenteritis rats compared with controls (P < 0.01). The incidence of bacterial translocation in rats that previously had enteritis was 54%, significantly greater (P < 0.05) than the 23% incidence in naive controls (Table 2).

Colonic epithelial proliferation. The average number of BrdU-positive cells per crypt-villus unit in untreated control rats was 19.2 ± 1.9 (n = 13), and that in rats treated intracolonically with 50% ethanol 6 wk earlier was 20.7 ± 2.0 (n = 6). Rats examined 6 wk after intracolonic administration of TNBS exhibited a significant increase in BrdU labeling (35.4 ± 4.0; n = 6; P < 0.05), whereas in rats that were treated 6 wk earlier with the ethanol vehicle, the numbers of BrdU-positive cells per crypt-villus unit were similar to those in untreated controls (20.7 ± 2.0; n = 6). Treatment with L-NIL for 1 wk did not significantly affect the rate of epithelial proliferation in postcolitis rats (38.0 ± 4.1 positive cells per crypt-villus unit vs. 34.5 ± 2.7 in vehicle-treated controls; n = 6–10 per group).

### Table 2. Colonic bacterial levels and bacterial translocation in postenteritis rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Total Colonic Bacterial Levels, log_{10} CFU/g</th>
<th>Colonic Gram-Negative Bacterial Levels, log_{10} CFU/g</th>
<th>Incidence of Bacterial Translocation, %</th>
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</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>13</td>
<td>6.8 ± 0.4</td>
<td>4.7 ± 0.6</td>
<td>23</td>
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<tr>
<td>Postenteritis</td>
<td>13</td>
<td>7.8 ± 0.6*</td>
<td>6.1 ± 0.6†</td>
<td>54*</td>
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</tbody>
</table>

Values are means ± SE. Studies were performed in male Sprague-Dawley rats killed 52 days after infection with *Nippostrongylus brasiliensis*. Bacterial colonization was measured in samples of the colonic tissue plus the luminal contents. Bacterial translocation was determined as described in MATERIALS AND METHODS. *P < 0.05, †P < 0.01 vs. control.

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**DISCUSSION**

The epithelium of the gastrointestinal tract forms an important barrier, limiting the entry of luminal microbes and toxins into the lamina propria. Epithelial secretion of electrolytes, water, and mucus may also participate in this aspect of host defense. For example, epithelial secretion has been suggested to play a role in clearing antigenic material from the intestinal lumen, diluting toxins, and limiting bacterial colonization (10). During inflammatory reactions, epithelial secretion may be altered as a result of the release of soluble mediators such as prostaglandins, NO, and various cytokines. However, following the resolution of inflammation, epithelial dysfunction can persist for prolonged periods of time. We have previously demonstrated that epithelial dysfunction in vitro persists for at least 12 wk after the episode of inflammation (TNBS-induced colitis), but this dysfunction was not seen in rats given only the ethanol vehicle (1). In the present study, we have extended this observation by demonstrating that colonic epithelial hyporesponsiveness can be observed in vivo for at least 6 wk after induction of colitis and also after resolution of small intestinal inflammation. The secretory dysfunction was a generalized effect, rather than being specific to a single stimulus. Thus the hyporesponsiveness was observed in vivo with two secretagogues that have distinct mechanisms of action (*C. difficile* toxin A and IBMX), and secretory dysfunction in vitro was demonstrated in a model of antigen-induced epithelial chloride secretion. Moreover, the rats that had previously been subjected to colitis or enteritis also exhibited significant increases in bacterial colonization of the colon, consistent with the notion that colonic epithelial secretion normally reduces bacterial colonization. The impairment of colonic secretion may also have contributed to the significant increase in bacterial translocation in the rats that previously had experienced a bout of colitis or enteritis. Interestingly, the tripling of the rate of bacterial translocation in postcolitis rats occurred despite the gastrointestinal epithelial permeability to a small molecular weight probe (EDTA) not being significantly different from that in normal rats. The fact that we have observed a marked increase in bacterial translocation in the absence of any detectable change in permeability suggests that caution should be exercised in drawing conclusions from studies that use...
permeability probes as a sole index of “barrier function.”

The results of the present study suggest a key role for iNOS-derived NO in the mechanism underlying the bacterial translocation and, to a lesser extent, epithelial hyporesponsiveness following a bout of colitis. The reversal of the postcolitis bacterial translocation by treatment with a selective iNOS inhibitor is consistent with a number of previous studies that suggested that iNOS-derived NO played a role in endotoxin- or ischemia-reperfusion-induced bacterial translocation in the intestine (11, 24, 46), although a molecular mechanism of action has not been identified. In previous in vitro studies in which epithelial hyporesponsiveness persisted for >12 wk after a bout of colitis, inhibition of iNOS was found to normalize the responsiveness of the colonic epithelium to electrical field stimulation and IBMX but not to carbachol (1). On the basis of these data, we proposed that NO might contribute to the hyporesponsiveness by affecting cAMP-mediated (such as that activated by carbachol) pathways of secretion. Recent studies by Freeman et al. (14) suggest that NO-mediated enterocyte hyporesponsiveness may be due to effects mediated at the level of adenylate cyclase. Our in vivo findings in the present study demonstrate that L-NIL treatment only partially reversed the epithelial hyporesponsiveness to IBMX and had no effect on C. difficile toxin A-stimulated secretion. C. difficile toxin A is believed to elicit its secretagogue effects through calcium-dependent pathways (19, 40), and previously it was demonstrated that an inhibitor of iNOS (aminoguanidine) had no effect on the secretory response to this toxin (41). C. difficile toxin A has also been shown to elicit epithelial toxicity. Consequently, it was possible that the difference in fluid accumulation levels observed between naïve and postcolitis rats could have been due to increased susceptibility to toxin A-induced damage in the latter group. However, cellular toxicity (as determined by lactate dehydrogenase release) in toxin A-treated vs. vehicle-treated colonic loops from naïve and postcolitis rats was not significantly different.

As mentioned above, acute administration of L-NIL (iNOS inhibitor) resulted in a partial reversal of the hyporesponsiveness to stimulation with IBMX. This indicates that the epithelial dysfunction was not attributable simply to an absence of the cellular apparatus for active chloride secretion (e.g., loss of expression of the chloride transporter). The results of the present study are consistent with the hypothesis that the defect lies at the level of the enterocyte rather than in cells in the lamina propria that can influence epithelial chloride secretion (e.g., nerves, mast cells). For example, in both the present study and in our previous in vitro study, we observed that the hyporesponsiveness was evident with a number of different secretagogues that act through different pathways. Moreover, in the present study we observed a complete absence of an epithelial secretory response to antigen in sensitized, postcolitis rats despite the fact that the antigen still caused mucosal mast cell degranulation. Finally, the observation that TTX did not reverse the hyporesponsiveness to IBMX suggests that changes produced by a prior bout of colitis are not attributable to alterations in the neural elements that regulate epithelial secretion.

In addition to the long-term changes in epithelial secretory function and bacterial translocation after a bout of colitis, we observed a significant increase in bacterial colonization and in the proliferation of the colonic epithelium. Unlike the bacterial translocation and secretory dysfunction (at least in response to IBMX), bacterial colonization levels and rates of colonocyte proliferation were not significantly affected by treatment with L-NIL. If the increase in bacterial colonization occurred as a consequence of impaired colonic secretion, then the lack of effect of treatment with L-NIL on bacterial numbers is not surprising. As discussed above, only a portion of the hyporesponsiveness appeared to be NO dependent. Further studies are needed to more fully comprehend the mechanisms responsible for colonic hyposecretion after colitis, which will then permit studies to determine the extent to which the changes in bacterial translocation (and possibly proliferation) are a consequence of the impaired secretory responsiveness.

The findings of this study may have important implications for understanding the pathogenesis of inflammatory bowel disease and irritable bowel syndrome. Intestinal infection has been suggested to be one of the possible triggers of inflammatory bowel disease (13). Several studies have reported that in a significant percentage of patients with irritable bowel syndrome, the symptoms can be traced to a bout of intestinal infection (17, 27, 32, 42). The present study has demonstrated that a bout of intestinal inflammation can cause long-lasting changes in epithelial function (secretion and proliferation) that could contribute to an increased propensity for infection and/or inflammation. There is also evidence of long-term changes in nerve and muscle function after a bout of colitis in humans and in animal models (16, 21, 47, 50). In addition, myenteric nerve function has been shown to be altered not only at the site of the inflammatory reaction but also at more distant, noninflamed sites (20, 31). These changes, along with the hyporesponsiveness of the epithelium, could contribute not only to reduced mucosal defense but also to the generation of symptoms (diarrhea, pain) in patients with inflammatory bowel disease or irritable bowel syndrome.

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REFERENCES


