In Vitro Model of Intestinal Crypt Abscess
A Novel Neutrophil-derived Secretagogue Activity

Shirin Nash, Charles Parkos, Asma Nusrat, Charlene Delp, and James L. Madara
*Division of Gastrointestinal Pathology, Departments of Pathology, Brigham and Women's Hospital and
Harvard Medical School, and the Harvard Digestive Diseases Center, Boston, Massachusetts 02115*

Abstract

In order to model crypt abscesses, a histological finding which correlates with disease activity in intestinal inflammation, human polymorphonuclear leukocytes (PMN) were layered onto monolayers of the human intestinal epithelial cell line T84, a crypt-like epithelium which is capable of Cl^- secretion. Such PMN-epithelial interaction had no substantial effect on monolayer integrity or function. However, when PMN were stimulated by conditions including those present naturally in the human colonic lumen, monolayers responded with a bumetanide-sensitive short circuit current (Isc) indicative of Cl^- secretion, the basis of secretory diarrhea. This Isc response was induced by a neutrophil-derived secretagogue (NDS), which was only active when applied to the luminal surface of monolayers and did not require PMN-epithelial contact. NDS activity is resistant to boiling, acid, and trypsin and passes a 500 nominal mol wt cutoff filter. NDS activity is not secondary to the respiratory burst products O_2^- or H_2O_2 and does not appear to be a myeloperoxidase product. We speculate NDS elicited Cl^- secretion may contribute to the secretory diarrhea seen in patients with intestinal inflammation and crypt abscesses. (*J. Clin. Invest.* 1991. 87:1474–1477.) Key words: Cl^- secretion • epithelium • inflammation

Introduction

Although inflammatory diseases of the intestine are, as a group, diverse, many of these diseases express similar morphologic features. For example, in active intestinal inflammation, polymorphonuclear leukocytes (PMN)[1] characteristically migrate across the epithelium to collect in crypts—a diagnostic feature, termed a "crypt abscess," which is used clinically to evaluate the level of activity of various inflammatory bowel diseases (1, 2). Using the T84 cryptlike intestinal epithelial cell line (3–5), and purified PMN, we have previously modeled this transmigratory event (6). Here we have modeled the crypt abscess by layering PMN on T84 monolayers and have assessed the functional significance of such PMN-T84 cell interactions. Present within the intestinal and crypt lumen are bacterial products such as lipopolysaccharide (LPS) (at approximate microgram-per-milliliter concentrations) (7) and n-formylated peptides (at approximate micromolar concentrations) (8). As modeled by FMLP (N-formyl methionyl leucyl phenylalanine), n-formylated peptides induce both respiratory burst activity (9) and degranulation (10) in PMN responses which may be enhanced when PMN are "primed" by LPS (11) as would be the case in the intestinal lumen. Thus, the known conditions in the intestinal lumen should affect the metabolic state of PMN within crypt abscesses, specifically, by inducing PMN to generate a complex array of reactive oxygen species, proteases, lipoxygenase, and other products which might influence the functional state of crypt epithelial cells. We have stimulated intact PMN in the above crypt abscess model to characterize PMN products which might effect T84 monolayer function. We find that luminal PMN, stimulated either by LPS and FMLP or by phorbol myristate acetate (PMA), do not substantially alter monolayer integrity. However, such stimulation does induce a bumetanide-sensitive short circuit current (Isc) in T84 cells which, in this cell line, is indicative of electrogenic Cl^- secretion, the cellular event which is the basis of secretory diarrhea (12). The Isc induced by activated PMN does not require epithelial-PMN contact and is mediated by a soluble product. This neutrophil-derived secretagogue (NDS) does not appear to be either H_2O_2 or monochloramine, PMN products which by themselves may induce intestinal Cl^- secretion in natural intestinal epithelia (H_2O_2) or in T84 monolayers (monochloramine) (13, 14). Preliminary physical data suggest NDS is not a lipid, but is small (≤ 500 mol wt) and is heat and acid stable. Along with the heat stable toxin of *Escherichia coli*, NDS is unique in inducing the Cl^- secretory response only when added to the apical side of monolayers.

1. *Abbreviations used in this paper:* LPS, lipopolysaccharide; NDS, neutrophil-derived secretagogue; PMN, polymorphonuclear leukocyte.

Methods

Confluent monolayers of the human intestinal epithelial cell line T84, were grown on collagen-coated permeable supports and maintained until steady-state resistance to passive transepithelial ion flow was achieved as previously described (4, 6, 15). Such monolayers are composed of cells uniformly adjoined to their neighbors by circumferential tight junctions which dramatically restrict the passive paracellular flow of ions and solutes (4, 6, 15). These monolayers serve as models for studies of electrogenic Cl^- secretion (3, 5). Transepithelial measurement of resistance to passive ion flow and of short-circuit current were performed as previously described (4, 6, 15). In initial experiments, in which PMN were layered onto T84 monolayers, a modified Ussing-type chamber, which allowed horizontal positioning of monolayers,

was used as we have previously described (15). For analyses of soluble neutrophil products on epithelial function, both vertically (4, 6) and horizontally mounted monolayers were studied. For the majority of these latter experiments we utilized monolayers prepared as above but having a surface area of 0.33 cm² an apical bath of 0.2 ml and a serosal bath of 0.8 ml. This was necessary to circumvent an otherwise prohibitive need for purified PMN. We verified the reproducibility of electrical data obtained from this system and the similarity of electrical data obtained from these monolayers to that of 2 cm² monolayers (data not shown).

PMN were purified from whole blood collected by venipuncture from normal donors of both sexes using the density sedimentation technique previously described (6). The PMN collection procedure was approved by the Brigham and Women's Hospital Human Subjects Committee (protocol 87-1465). PMN (95% pure) with 98% viability by dye exclusion were suspended in tissue culture medium or in HBSS at 4°C, and were used for experiments within 1 h after isolation. H_2O_2 was measured as H_2O_2 production at 25 min in a microtiter assay, as described by Pick and co-workers (16, 17).

In subsets of experiments, supernatants from stimulated PMN were precipitated with two parts ethanol at 4°C for 30 min and centrifuged at 9,000–10,000 g for 9–20 min. After removing the supernate, the resulting precipitates were resuspended to the initial volume from which they were obtained in HBSS. Resuspended precipitates were loaded onto solid phase extraction cartridges (C18 Seppaks; Millipore Corp., Milford, MA) and the stationary phase was sequentially eluted with hexane, methyl formate, and methanol as described by Serhan and Sheppard (18).

All tissue culture reagents were obtained from Gibco (Grand Island, NY). Bumetanide was obtained from Hoffmann LaRoche, Nutley, NJ, superoxide dismutase and catalase from Worthington Biochemicals, St. Louis, MO, TIMP from Synergen, Boulder, CO, and all other reagents from Sigma Chemical Co., St. Louis, MO.

Results

Approximately 400 monolayers were used in these studies.

PMN activation elicits Isc response from T84 cells. Stimulation of PMN, as determined by H_2O_2 production, was achieved with 0.1 µg/ml of PMA in HBSS (63.3±5.7 nmol $H_2O_2/10^6$ PMN, n = 24). In subsets of experiments in which 1 mg/ml BSA was present, 1 µg/ml PMA was needed to achieve the same result due to protein sequestration of PMA. However, the presence or absence of protein did not influence the results to be presented below. PMN primed by endotoxin (LPS, 0.1–1 µg/ml, 30–45 min) and subsequently exposed to FMLP (10^{-6} M) produced H_2O_2 of 6.8 nmol/10^6 PMN (n = 16). The above concentrations of LPS, FMLP, and PMA were used for subsequent experiments.

LPS-FMLP or PMA in the doses used to activate PMN had no substantial effect on monolayer resistance or peak short-circuit-current (resistance, 860±24, 795±38, 825±22 ohm cm²; Isc, 0.6±0.2, 1.9±0.4, 0.7±0.4 µA cm^{-2} for control, PMA exposed, or LPS-FMLP exposed monolayers, respectively, all n = 6–10). Similarly, layering unstimulated PMN on T84 cells (5×10^6 PMN · cm^{-2}) had minimal effect on monolayer Isc (Fig. 1), and on monolayer resistance (812±42 vs. 780±30 Ωcm² for monolayers without vs. monolayers with PMN at 20 min). In contrast, LPS-FMLP or PMA stimulation of PMN in the luminal compartment elicited an Isc which peaked at 10–20 min after stimulation but was detectable within 2 min. In the subgroup of experiments from which time course data were initially collected (Fig. 1), the average peak Isc response was ~ 6 µA · cm^{-2}. The peak response in a large sample of experiments performed in this fashion (PMN first layered onto monolayers, n = 52) was 10.5±0.2 µA · cm^{-2}. Our extended data which also
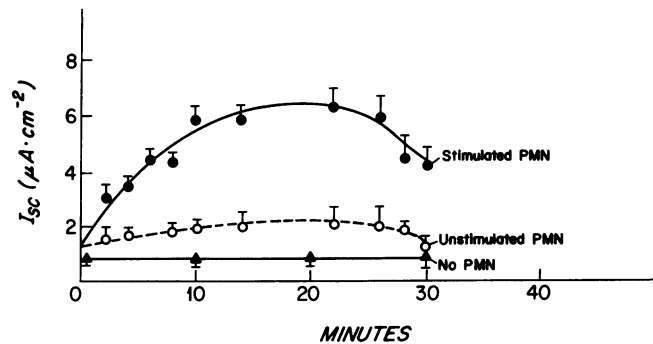
Figure 1. Isc response of T84 monolayers without PMN, or with stimulated or unstimulated PMN layered on the apical surface (5 × 10^6 PMN-cm^{-2}). Stimulated PMN elicited a Isc response detectable within 2 min. Although the agonist used here was PMA, comparable results were obtained with LPS-FMLP (see text for doses and description). Such agonists when applied to T84 cells in the absence of PMN had not significant effect on Isc (see text). (all n = 7–12)

includes experiments utilizing either PMN or PMN-derived supernatants (to follow below) showed that the peak Isc response varies from ~ 4–50 µA · cm^{-2} (n ~ 250). The Isc response did not vary with the method of PMN stimulation (PMA vs. LPS-FMLP, data not shown).

To elicit the above described Isc, stimulated PMN did not need to be present in great excess to T84 cells. As shown in Fig. 2, > 50% of the maximal Isc response occurred when stimulated PMN were present at equal numbers to T84 cells. A greater Isc response (84±16%) was elicited by PMN:T84 cell ratios of 3:1. In these experiments a PMN:T84 cell ratio of 10:1 was defined as the maximal response both because a PMN:T84 ratio of 20:1 does not elicit a greater response and, as will be shown in the preliminary characterization studies described below, a 3× concentrate of the neutrophil-derived secretagogue (NDS) obtained at the 10:1 density also does not further increase the Isc response. In contrast, both the PMN vehicle (buffer + activator) or unstimulated PMN had little effect on Isc (Fig. 2).

The PMN-elicited Isc response does not require PMN-T84 cell contact. Contact between PMN and T84 cells was not required for the Isc response elicited by PMN activation. PMN separated from T84 cells by an interposed nucleopore filter still elicited an Isc response when stimulated, as did PMN from a patient in leukocyte adhesion deficiency (19) (data not shown).
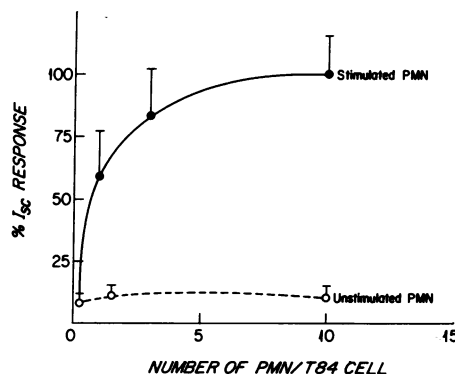
Figure 2. Isc response elicited by stimulation of PMN as function of varying PMN:T84 cell ratio. The Isc response can be detected at a PMN:T84 cell ratio of 1:1. PMN:epithelial cell ratios in crypt abscesses in human disease were counted by one of the authors (SN) and were found to fall within the range of ratios displayed in the figure (all n = 4–9).

Furthermore, when PMN were activated in the absence of T84 cells, removed by centrifugation, and the PMN-free supernate placed on T84 monolayers, the Isc response was again obtained (8.7 ± 1.9 vs. 0.4 ± 0.3 $\mu A \cdot cm^{-2}$ for supernatant vs. control, $P < 0.01$). These data indicate that PMN-T84 cell contact is not necessary for the Isc response and a soluble mediator is involved in this response.

Neutrophil-derived secretagogue (NDS) acts apically and is inhibited by bumetanide. As shown in Fig. 3, no NDS activity, as assayed by the Isc response, was detected when NDS was applied basolaterally. Also (Fig. 3) by preincubating monolayers with serosal bumetanide, (10^{-4} M) for 20–30′, the Isc response to apically applied NDS was largely (> 85%) inhibited ($P < 0.01$) indicating that the Isc response obtained was due to Cl^- secretion (5).

NDS is not H_2O_2 (although H_2O_2 elicits Isc in T84 monolayers). Because Powell and co-workers, using mammalian intestinal mucosa, have shown that H_2O_2 is a Cl^- secretagogue (13), we sought to determine if NDS represented H_2O_2. As shown in Fig. 4, H_2O_2 can also elicit an Isc in T84 monolayers. However, no sustained Isc response is noted with 1 mM H_2O_2 (or with 0.1 mM, not shown) and with 10 mM H_2O_2 the maximum response is far less than the NDS response obtained in parallel experiments (4.4 ± 0.5 vs. 17.5 ± 2.9 μA cm^{-2} for 10 mM H_2O_2 vs. NDS, respectively, $P < 0.01$). The NDS containing supernate used in this experiment was incubated at 20°C for 1 h before the experiment, conditions which led to a loss of H_2O_2 (H_2O_2 of this NDS supernate $< 10^{-4}$ M). In agreement with these findings, as shown in Table I, NDS activity was also not ablated when the supernatant was generated in the presence of superoxide dismutase and catalase.

We also examined the effects of other inhibitors of neutrophil products or metabolic pathways on the ability of PMN to generate NDS activity when stimulated. As shown in Table I, none of the inhibitors tested significantly attenuated NDS activity (PMN were preincubated with all inhibitors before stimulation). Pretreatment of PMN with cytochalasin B (5 $\mu g/ml$) for 30 min before stimulation with PMA also did not enhance NDS activity. These latter conditions potentiate PMN degranulation upon subsequent stimulation by soluble agonists (21) and thus such data suggest NDS is not a product contained in PMN granules. Lastly, as shown in Table I, the failure of protease inhibitors to ablate NDS activity in supernates of stimu-
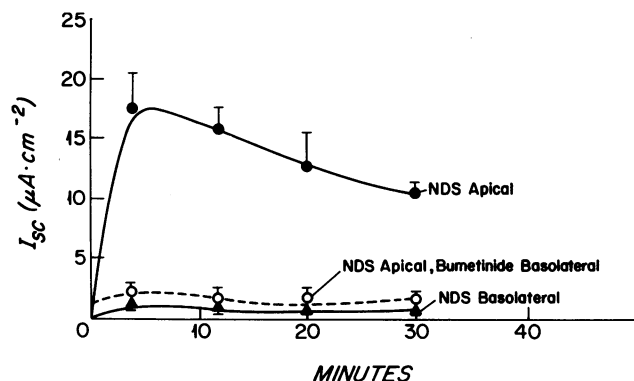
Figure 3. Isc responses to cell free supernates obtained from stimulated PMN. The neutrophil-derived secretagogue (NDS) activity in the PMN supernate is not active when added to the basolateral side of the monolayers. Bumetinide (10^{-4} M, serosal) inhibits 90% of the NDS response (all $n = 6$–10).
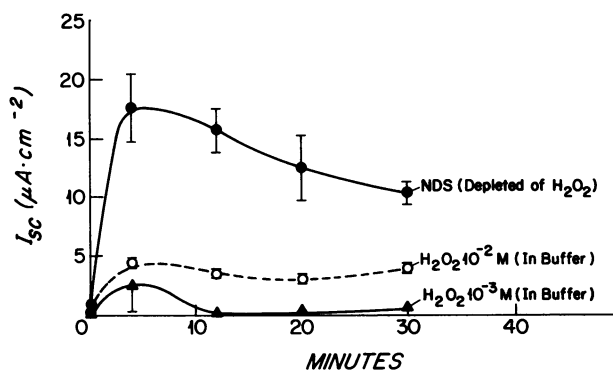
Figure 4. Isc response to H_2O_2 or NDS depleted of H_2O_2. H_2O_2 will elicit a small Isc response when added to the mucosal bath at 10^{-2} M. Lower concentrations of H_2O_2 (including 10^{-4} M, not shown) have no substantial effects on Isc. However, NDS when depleted of H_2O_2 elicits its usual Isc response. As described in the text, NDS activity was also unaffected when PMN supernates were generated in the presence of SOD and catalase. Thus, while it is possible that H_2O_2 at high concentration may elicit a very small Isc response directly, NDS activity is not due to H_2O_2 (all $n = 6$–9).

lated PMN, suggests that NDS activity is likely not due to proteolysis of another PMN product nor due to the effects of PMN-derived proteases on the apical membrane of T84 cells. In agreement with this latter point, NDS is smaller than PMN-derived proteases (see below).

Preliminary analyses of physical characteristics of NDS. NDS activity was not ablated by either 1 μM trypsin or 1 μM chymotrypsin for 2 h at 37°C (83 ± 12 and $87 \pm 9\%$ of control value); was not lost after 40 min boiling ($96 \pm 10\%$ of control value); was not sensitive to 0.1% trifluroacetic acid (70% of control value); and withstood lyophilization and resuspension (92% vs. control value). In four experiments, 41–70% of NDS

Table I. Inhibitors of PMN Metabolic Pathways Do Not Diminish Isc Response to NDS

Inhibitor	Product effected	Loss of NDS activity
		%
Sodium azide, 1 mM ($n = 17$)	Myeloperoxidase products (i.e., hypochlorite monochloramine)	0 (NS)
Catalase, 5000 U/ml ($n = 4$)	H_2O_2	10 ± 7 (NS)
Catalase$^+$ SOD, 5000 + 280 U/ml ($n = 11$)	Reactive oxygen species	0 (NS)
DFP, 5 mM ($n = 6$)	Serine proteases (i.e., elastase, cathepsin G, proteinase 3)	29 ± 14 (NS)
Heparin 10 $\mu g/ml$ ($n = 5$)	Heparinase	25 ± 9 (NS)
TIMP 20 $\mu g/ml$ ($n = 8$)	Metaloproteinases	24 ± 7 (NS)
Mepacrine 150 μM ($n = 7$)	Phospholipase A2 (cyclooxygenase, lipogenase products)	0 (NS)
Esculatine 100 μM ($n = 5$)	Lipooxygenases (12LO > 5LO)	0 (NS)

activity, as defined by the Isc response, could be recovered from ethanol precipitates of the PMN supernate. However, unlike NDS activity in a PMN supernate, the NDS activity of the resuspended pellet was largely lost at a 3× dilution, indicating that only a small amount of the compound(s) responsible for NDS activity were precipitated out. NDS activity also does not appear to be associated with a highly hydrophobic compound(s) such as a phospholipid, leukotriene, prostaglandin or peptidolipid: NDS activity could not be extracted by hexane, methyl formate or methanol (two experiments, each with $n = 3-6$ for each solvent; all solvent extracts contained < 8% of NDS activity). Lastly, $94\pm8\%$ ($n = 12$) of NDS activity passed through an ultrafiltration device with a 500 nominal molecular weight cut off, indicating the NDS activity resides within a pool of rather small compound(s).

Discussion

We show that PMN stimulated by PMA or LPS-FMLP, release a soluble small, heat and acid stable, hydrophilic, and trypsin-resistant compound which acts apically on T84 cells to elicit a bumetinide-sensitive Isc, indicative of Cl^- secretion. Although H_2O_2 can elicit Cl^- secretion when applied to intestinal mucosa (13), the Isc response generated by H_2O_2 in T84 cells is less than that of NDS. Also, NDS activity is not sensitive to catalase, and supernates from activated PMN which have been depleted of H_2O_2 have full NDS activity. Recently, monochloramine, a myeloperoxidase product of PMN, has been shown to elicit Cl^- secretion in T84 monolayers (14). However, in contrast to this small compound, NDS is active only when applied to the luminal side of T84 cells. Furthermore, NDS activity is not attenuated when myeloperoxidase activity of PMN is inhibited before PMN activation. Adenosine, another potential PMN product, also stimulates Cl^- secretion but, in contrast to NDS, does so efficiently whether applied apically or basolaterally (20). Additionally, NDS activity is unattenuated if PMN are treated with a lipoxygenase inhibitor before stimulation, and extraction of NDS containing precipitates with solvents known to isolate lipoxygenase products (18) does not isolate any NDS activity. Lastly, because LPS-FMLP-stimulated PMN generated NDS activity as efficiently as PMA-stimulated PMN, the NDS-stimulated Isc cannot be attributed to a PMN derived factor active only if T84 cells are "primed" with phorbol ester. In current experiments we are attempting to purify and identify the neutrophil product(s) associated with NDS activity.

Of interest is that the neutrophil:epithelial cell ratios used in this study are well within the range of those actually found in crypt abscesses (6) and that the conditions for PMN activation by LPS-FMLP exist within the colonic lumen (7, 8). Our results in T84 cells suggest that PMN in intestinal crypt abscess may release a novel secretagogue(s) active only from the apical membrane of the cell. To our knowledge, the only other known Cl^- secretagogue which is effective from the apical, but not the basolateral pole of intestinal epithelia or T84 cells is a compound far larger than NDS, heat stable toxin of *E. coli* (22). We speculate NDS may contribute to diarrheal disease seen in states of acute intestinal inflammation.

Acknowledgments

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