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References

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Apical Secretion of a Pathogen-Elicited Epithelial Chemoattractant Activity in Response to Surface Colonization of Intestinal Epithelia by Salmonella typhimurium

Beth A. McCormick,²*†§ Charles A. Parkos,*‡§ Sean P. Colgan,‡§ Denice K. Carnes,* and James L. Madara*†§

Modeling Salmonella-epithelial cell interaction in vitro has led to the realization that epithelial cells are crucial in orchestrating neutrophil (PMN) responses, in part by stimulating basolateral release of epithelial chemokines, including IL-8. However, such basolaterally released chemokines, while likely important in orchestration of PMN movement across the subepithelial matrix, are unlikely to be responsible for the final step of transepithelial migration of PMN and entry into the apical compartment. We now show that S. typhimurium attachment to T84 cell apical epithelial membranes induces polarized apical secretion of a pathogen-elicited epithelial chemoattractant (PEEC) bioactivity. Experiments employing semipurified PEEC indicate that it is released in a polarized apical fashion and is sufficient to explain the observed final step of transepithelial migration of PMN induced by Salmonella-apical membrane interaction. By preliminary physical characterization and profiles of PMN activation, PEEC appears to be a novel PMN chemotactic bioactivity. This 1- to 3-kDa nominal molecular mass chemokine-like bioactivity directly stimulates PMN via a pertussis toxin-sensitive receptor and elicits a Ca²⁺ signal. While these latter features are shared by most other chemokines, analysis of PEEC-elicited PMN activation reveals that, unlike these other agonists, PEEC, even at saturating concentrations, elicits chemotactic activity in the absence of stimulation of superoxide production and/or release of primary and/or secondary granules. These data suggest that the apically released PEEC activity appears to represent a novel epithelial-derivative chemoattractant that directs PMN movement across epithelial monolayers. The Journal of Immunology, 1998, 160: 455–466.

The epithelial lining of the intestine exemplifies a site at which host defense against environmental pathogens is paramount for survival. During intestinal disease induced by Salmonella typhimurium, transepithelial migration of polymorphonuclear leukocytes (PMN) rapidly follows attachment of the bacteria to the epithelial apical membrane (1–3). Thus, transepithelial migration of PMN occurs early after Salmonella-epithelial contact (3), and well before the epithelium loses its structural integrity (1). While such observations imply that interactions between intestinal epithelial cells and S. typhimurium play a key role in orchestrating the inflammatory response (4–8), understanding of mechanisms responsible for coordinating mucosal inflammatory responses to such pathogens remains incomplete.

Recent studies show that S. typhimurium contact with the apical pole of intestinal epithelial cells generates signal(s) that may be responsible for directing the trafficking of neutrophils across the intestinal epithelium. For example, we have previously demonstrated, using in vitro models of intestinal inflammation, that apical attachment of S. typhimurium to intestinal epithelial monolayers spontaneously stimulates physiologically directed PMN transepithelial migration. The signals responsible for orchestration of this inflammatory response do not utilize PMN N-formyl peptide receptor-directed migration (6), the best-understood, bacterial-derived, receptor-mediated pathway for directing PMN to a bacterial target. Among the events stimulated by such pathogen-host interactions is the release of chemotaxins that might guide PMN to the site of bacterial-epithelial contact (5–8). For example, S. typhimurium-intestinal epithelial cell interactions induce the epithelial synthesis and polarized basolateral release of the potent neutrophil chemotactic peptide IL-8 (4–7). However, while such basolateral secretion of IL-8 can direct PMN through the underlying matrix and to a subepithelial position via matrix imprinting (4), basolateral release of this chemokine is insufficient to induce migration across the epithelium (6). This later finding is not surprising given that IL-8 released from the basolateral surface of the epithelium results in an inappropriately directed (basolateral > apical) gradient to support transepithelial movement of PMN. Additionally, we recently provided evidence that transepithelial signaling events elicited by S. typhimurium-apical membrane contact represents a key virulence mechanism by which enteritis occurs in vivo (5). Here we show that S. typhimurium interactions with the apical plasma membrane of intestinal epithelial cells also elicit apically polarized secretion of a unique
bioactivity with potent PMN chemotactic activity. Transfer studies indicate that this apically secreted chemooattractant is highly efficient in driving PMN migration across the epithelium (the final step of transepithelial migration).

In this study we examine the physical/biologic characteristics of PEEC (pathogen-elicted epithelial chemooattractant). The distinguishing features of PEEC indicate that it is relatively small (nominal 1–3 kDa; unlike many known chemokines, stable, not highly hydrophobic, and does not appear to signal via the n-formyl peptide or IL-8 receptors. Like most neutrophil chemooattractants, PEEC induces a PMN signal transduction cascade involving a GTP binding protein (Gq) that also elicits a rise in [Ca2+]i. In addition, bioassays reveal that PEEC directly signals PMN but, also unlike known chemooattractants, PMN respond to PEEC with essentially a purely chemotactic response in which degradation or superoxide generation is virtually not detectable, even at saturating concentrations of PEEC bioactivity. Importantly, these data predicate that epithelial cells can respond to surface pathogens such as Salmonella by releasing distinctive chemooattractants that sequentially orchestrate movement of PMN into the subepithelial compartment by matrix imprinting (i.e., IL-8), and as shown in this report, subsequently guide PMN across epithelia into the luminal compartment (i.e., PEEC).

**Materials and Methods**

**Cell culture**

T84 intestinal epithelial cells (passages 70–95) were grown and maintained as confluent monolayers on collagen-coated permeable supports (9) with recently detailed modifications (10). Monolayers were grown on 5-cm² and 0.33-cm² suspended polycarbonate filters (Costar Corp., Cambridge, MA) and utilized 7 to 14 days after plating, as described previously (10). A steady-state resistance (approximately 1500 ohm cm²) is reached in 5 days with variability largely related to cell passage number. Inverted monolayers used to study transmigration of neutrophils in the physiologic basolateral-to-apical direction were constructed as described before (10–12).

**Bacterial strains and growth conditions.** S. typhimurium χ306 is a naladixic acid-resistant ( gyrA1816) strain derived from S. typhimurium strain SR-11 (13). Luria broth was made as previously described by R evil (14). L. agar is Luria broth containing 12 g of Bacto Agar (Difco Laboratories, Detroit, MI) per liter. Bacterial growth conditions were as follows: non-agitated microaerophilic bacterial cultures were prepared by inoculating 10 ml of Luria broth with 0.01 ml of a stationary-phase culture followed by overnight incubation (approximately 18 h) at 37°C, as previously detailed (6, 15).

**Transcellular chemotactic factor (PEEC) collection.** S. typhimurium interactions with polarized T84 apical surfaces were as follows: to harvest PEEC bioactivity, polarized T84 monolayers grown on 5-cm² inserts were subsequently infected by S. typhimurium suspended in HBSS + buffer, performed by the method of McCormick et al. (6), as shown in Figure 1A, with a slight modification. Briefly, T84 monolayers (10–12) were extensively rinsed in HBSS + (containing Ca2+ and Mg2+), with 10 mM HEPES, pH 7.4 (Sigma Chemical Co., St. Louis, MO) to remove residual media and serum components. Approximately 5 × 10⁶ bacteria in 1.0 ml HBSS + was gently distributed onto the apical surface and incubated for 45 min at 37°C. Non-adherent bacteria were subsequently removed from the monolayers by extensive washing (four times with 4.0 ml of buffer/wash) and were then transferred into the six-well tissue culture tray containing HBSS + buffer (in each lower (basolateral) and upper (apical) reservoir (apical surface now colonized with S. typhimurium). Following an incubation of 2 h at 37°C, both the apical and basolateral supernatants were collected, filtered through a 0.2-μm filter, and stored at −80°C until further use. In the subset of experiments to determine the time course of PEEC secretion, T84 monolayers were colonized by S. typhimurium, and subsequent to the removal of non-adherent bacteria, were incubated in HBSS + buffer for 2 h and the apical supernatant was removed. Fresh buffer was then added to these same monolayers, and incubated for an additional 2 h (4 total h). The apical supernatants were collected, filtered through a 0.2-μm filter, and stored at −80°C until further use. This cycle was repeated for an additional 2 h, corresponding to the 6-h time interval.

**PMN transepithelial migration.** PMN obtained from normal human volunteers were isolated from anticoagulated whole blood, as previously described (16, 17). A total of 1 × 10⁶ isolated PMN were added to the basolateral bath of inverted 0.33-cm² monolayers (upper reservoir equates with basolateral side since monolayers were grown in an inverted fashion). Incubations were for 120 min at 37°C unless specifically indicated otherwise. As shown in Figure 1B, the PEEC bioactivity of the collected supernatants was tested by placing harvested material (300–500 μl) in the lower (apical) compartment. Positive control transmigration assays were performed by imposing chemooattractant gradients (10⁻⁷ M FMLP) favoring basolateral-to-apical migration across inverted monolayers. At the end of each experiment PMN transmigration was quantified (as cell equivalents; CE) by assaying for the PMN-specific azurophilic granule marker myeloperoxidase as described previously (18). PMN (CE); were assessed as the number of PMN that had completely traversed the transepithelial monolayer (into the lower reservoir).

**Underagarose chemotaxis assay.** The underagarose assay was used to assess chemotaxis in the absence of an epithelial monolayer as detailed previously (19) using human peripheral blood PMN. Briefly, a mixture of 1% agarose (Sigma Chemical Corp.) and 0.5% gelatin (Life Technologies, Grand Island, NY) was solidified on 60-mm petri dishes (6 ml/plate). Eight sets of three 3-mm wells were punched in the plates with wells spaced 3 mm apart. Plates were preincubated with chemooattractant for 45 min at 37°C. Subsequently, addition of fresh chemooattractant, buffer controls, and
purified neutrophils (10 μl at 10^7/ml) were added to the corresponding wells of the migration plates. Plates were incubated for 120 min at 37°C in a humid chamber and fixed with 5% glutaraldehyde overnight. After removal of agarose gels, plates were rinsed and air dried. PMN migration was measured microscopically by the leading front of migrating PMN (18) utilizing an ocular micrometer at ×400. From each plate, directed migration was scored and the migration was measured. Data are presented as a ratio (chemotactic index).

**Analysis of PEEC physical characteristics.** To estimate the nominal m.w. of PEEC, the supernatants were passed through a series of filters with defined m.w. exclusion (10,000, 3,000, and 1,000; YM10, YM3, and YM1, respectively) using an Amicon filtration unit (model 8400; Amicon Inc., Beverly, MA). Each filter retentate (supernatant remaining after the filter) and filtrate (supernatant that passed through the filter) was collected and tested for the ability to elicit PMN transepithelial migration. Unless otherwise indicated, PEEC retained in the 1000-retenate fraction was concentrated 20-fold by volume. This volume concentration step was necessary since a larger amount of HBSS^−/cm^2 is required to accommodate the transwell system.

**PEEC stability.** 1) Temperature sensitivity: to determine whether the PEEC bioactivity was heat stable, PEEC was heated to 100°C for 20 min, and subsequently examined for the ability to induce PMN transepithelial migration. 2) Freeze/thaw: the factor was subjected to three freeze/thaw cycles and then tested for the ability to elicit PMN transepithelial migration. In addition, supernatants were vacuumed dried, resuspended to the initial volume from which they were obtained in HBSS, and stored at 4°C for 30 min. After centrifugation (2000 rpm), concentrated (50-fold) PEEC was added to two parts of absolute ethanol and placed at 4°C for 30 min. After centrifugation, the resulting supernatant and filtrate (supernatant that passed through the filter) was collected and tested for the ability to elicit PMN transepithelial migration. Unless otherwise indicated, PEEC retained in the 1000-retenate fraction was concentrated 20-fold by volume. This volume concentration step was necessary since a larger amount of HBSS^−/cm^2 is required to accommodate the transwell system.

**Phase separation/solubility studies.** In subsets of experiments, PEEC was precipitated with two parts of absolute ethanol at 4°C for 30 min, and centrifuged at 10,000 × g for 20 min (11). The supernatant and precipitate was individually processed to determine which fraction contained the bulk of bioactivity when tested for neutrophil transepithelial migration. The precipitate and the supernatant, following vacuum drying, were resuspended to the initial volume from which they were obtained in HBSS^−.

**Hydrophobic extraction.** To determine whether PEEC was highly hydrophobic, we employed a Sep-Pak extraction procedure (Waters Corp., Milford, MA). Concentrated (50-fold) PEEC was added to two parts of absolute ethanol and placed at 4°C for 30 min. After centrifugation (2000 rpm), the supernatant was collected in a round-bottom flask, and was obtained (4). Briefly, 50 μg/ml of the Ab was added to 5 × 10^5 PMN and the neutrophil-mAb mixture was gently rotated at 4°C for 90 min to prevent settling of the PMN. The enzyme control included PEEC in the absence of trypsin but was treated for 2 h at 37°C, followed by 15 min at 100°C. Further, PEEC was treated with 10 U proteinase K (Sigma Chemical Corp.) at 37°C for 60 min. The enzyme was deactivated by boiling for 15 min (a manipulation that does not affect PEEC activity; see Results). The vehicle control consisted of PEEC in the absence of proteinase K treatment, but included heat treatment at 37°C for 60 min followed by boiling for 15 min. Both PEEC-trypsin- and proteinase K-treated samples were tested for their ability to elicit PMN transepithelial migration.

**Results**

**Peptide synthesis or chemotaxis.** Calcium influx: human peripheral blood neutrophils at a density of 1.5 × 10^6 (50 μl of 5 × 10^7/ml stock) were attached to aceton-washed glass coverslips for 15 min at room temperature. Nonadherent PMN were then washed three times with normal buffer solution (140 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2, 1.1 mM MgCl_2, 2.6 mM dextrose, 10 mM HEPES, pH 7.4) and loaded with fura 2-AM (final concentration 7.5 μM) for 1 h at room temperature. The fura-loaded PMN were washed once in normal buffer solution, cooled on ice, and challenged with saturating concentrations (determined by initial peak of dose-response curve) of various chemoattractants that included n-FMLP (10^-8 M; Sigma), IL-8 (500 ng/ml; R&D Systems), and the test sample, PEEC (200-fold concentrated by volume representing the saturating concentration of this chemoattractant; see Results). Control buffer in this experiment is HBSS^-^-conditioned with T84 cells for 2 h at 37°C. Measurement of calcium influx was performed in a spectrofluorometer, and was reported as the difference in fluorescence between 510 and 530 nm (Photon Technology International, model 4000; S. Brunswick, NJ). [Ca^2+], (1 mM) was calculated according to the following equation:

\[
[Ca^{2+}] = \frac{F - F_{min}}{F_{max} - F_{min}}
\]

Where F is the observed fluorescence, F_{min} was obtained by saturating the intracellular dye with Cu^2+ using digitonin, and F_{max} from background fluorescence using HBSS^-.

**Neutrophil superoxide assays.** Superoxide generation from human PMN was measured as the superoxide dismutase-inhibitable reduction of cytochrome c as previously described (24), with some modifications. Assays were performed in a 96-well microtiter plate with each well containing 0.2 ml final volume. To each well was added a small volume of agonist including FMLP (2 μl of a 10-μM stock in HBSS; final concentration 100 nM), PMA (1 μl of a 1-mg/ml stock in DMSO; final concentration 500 ng/ml), IL-8 (10 μl of a 14-μM stock in HBSS; final concentration 0.7 μM), PEEC (100 μl of a 2000-fold filtrate in HBSS), or conditioned HBSS (control) to be determined. Each well was incubated for 5 min at 37°C before adding the addition of FMLP followed by the addition of 100 μl of cytochrome c buffer (1.25 mg cytochrome c (Sigma; horse heart type VI) per ml of HBSS) containing 1 × 10^7 PMN. Matching wells were prepared that also contained superoxide dismutase and catalase at a final concentration of 125 μg/ml (Calbiochem (La Jolla, CA) and Sigma, respectively). For cytochalasin P-1, PMN were resuspended in cytochrome c buffer, and were treated with 7 μg/ml of diphycytocyanin B for 3 min (20°C) before initiation of the assay. Assays were initiated by the addition of PMN in cytochrome
lateral from the apical compartment of Salmonella-colonized monolayers induced transepithelial migration of PMN when transferred to the apical compartment of unmanipulated monolayers. In contrast, other transfers (basolateral supernatant to apical compartment, apical supernatant to basolateral compartment, basolateral supernatant to basolateral compartment) did not result in PMN transepithelial migration.

Although we have previously shown that basolateral secretion of IL-8 occurs due to Salmonella colonization of the apical membrane (4, 6), we have also observed that the concentration of IL-8 secreted basolaterally is insufficient to drive transepithelial migration when the identical concentration is placed in the apical bath (data not shown). Thus it was not surprising that basolaterally transferred supernatants derived from Salmonella-colonized monolayers did not stimulate spontaneous migration of PMN when transferred to the apical reservoir of unmanipulated monolayers. Additionally, transfer of apical (or basolateral) supernatants from one set of epithelial monolayers (that had not been surface colonized with Salmonella) to another also failed to elicit spontaneous PMN transepithelial migration (0.212 ± 0.067 and 0.389 ± 0.051, CE 1 × 10^4 for apical and basolateral T84 cell-conditioned supernatants, respectively). To reconcile that this was not a cell line-specific event, S. typhimurium was also found to signal to neutrophils through polarized monolayers of Caco2 cells with a corresponding apical release of PEEC (data not shown).
endotoxin units/ml. This level of endotoxin is not dissimilar to the bioactivity defined as PEEC, we measured the levels of LPS in transmigration response.

apically transferred signal generally reproduced 20 to 40% of the sideral response. However, for any given experiment the size of the PMN transmigration response varied con- dent of PMN IL-8A and n-formyl peptide or human rIL-8 gradients FMLP, as shown in Fig. 2), did promote PMN transepithelial migration when transferred to either the basolateral or apical compartment (+, p < 0.05). Data are expressed as mean and SD of triplicate samples for each condition examined, and represent one of at least three experiments performed with similar results. A-A, apical supernatant placed in apical compartment; B-A, basolateral-collected supernatants placed in the apical compartment; A-B, apical supernatant placed in the basolateral compartment; B-B, basolateral supernatants placed in the basolateral compartment. Insert, The bioactivity from the apical compartment of Salmonella-colonized monolayers that supports spontaneous PMN transepithelial migration when transferred to virgin epithelial monolayers is independent of PMN n-formyl peptide and IL-8A receptor antagonists as used in Figure 2. Data are expressed as mean and SD of triplicate samples for each condition tested and represent one of three experiments performed with similar results. NS, not significantly different.

We next tested whether this soluble, transferable PEEC was, as found for the spontaneous PMN migration across Salmonella-colonized monolayers (Fig. 2), also independent of the PMN n-formyl peptide and IL-8A receptor signaling pathways. As shown in the inset to Figure 3, antagonists for these receptors (that were effec- tive in inhibiting transepithelial migration by imposed n-formyl peptide or human rIL-8 gradients FMLP, as shown in Fig. 2), did not affect transmigration in response to apical transfer of PEEC. Similar findings occurred when neutralizing polyclonal Abs to IL-8 (30 μg/ml, sufficient to inhibit responses to imposed gradients of human rIL-8) were utilized (data not shown). Taken together, these data indicate that, as a result of S. typhimurium-apical epithelial associations, a soluble bioactivity is released into the apical compartment. This bioactivity is sufficient to induce spontaneous transepithelial migration in the physiologic direction and, like transmigration in Salmonella-colonized monolayers, is independent of PMN IL-8A and n-formyl peptide receptors. Furthermore, although the size of the PMN transmigration response varied con- siderably with individual donors, for any given experiment the apically transferred signal generally reproduced 20 to 40% of the transmigration response.

To determine whether Salmonella-derived LPS could influence the bioactivity defined as PEEC, we measured the levels of LPS in the apical supernatants of monolayers that were surface colonized by Salmonella. Samples containing PEEC had LPS levels of <1.25 endotoxin units/ml. This level of endotoxin is not dissimilar from that trace amount found in HBSS+ buffer controls (<1.25 endotoxin units/ml). Moreover, purified LPS from S. typhimurium failed to stimulate neutrophil transepithelial migration when ex- amined across a three-log dose response, 0.1 μg/ml to 10 μg/ml (0.37 ± 0.027, 0.383 ± 0.028, 0.374 ± 0.021, vs 0.421 ± 0.350 and 19.83 ± 3.0 for 0.10 μg/ml, 1.0 μg/ml, 10.0 μg/ml LPS vs negative buffer control and 10−7 M FMLP, respectively). The dose range of LPS utilized represents the average concentration of LPS estimated from bacterial densities corresponding to conditions that the monolayers are exposed to during infection, and include both a one-log higher and one-log lower density than that that occurs at the monolayers. Likewise, isolated S. typhimurium outermembranes were also ineffective in inducing neutrophil transepithelial migration (data not shown). Thus, LPS does not appear to account for the bioactivity observed. Additionally, as will be shown below, PEEC bioactivity can be captured in a low molecular mass filtrate that excludes LPS (a large macromolecular structure with a weight >100,000 kDa). Thus, LPS is not required to obtain transferable PEEC bioactivity.

Production of apical PEEC requires Salmonella-T84 epithelial contact

We next determined whether S. typhimurium-epithelial apical contact, or merely apical exposure to soluble S. typhimurium products was required for production of transferable PEEC bioactivity. Exposure of monolayer apical membranes to S. typhimurium-soluble products obtained from bacterial densities ranging from 5 × 106 to 5 × 107 bacteria/ml were exposed to apical plasma membranes of polarized T84 cell monolayers and compared with conditions that allowed direct Salmonella-epithelial cell contact for their ability to induce neutrophil transepithelial migration. All supernatant preparations were concentrated 20-fold (by volume) over a 1-kDa molecular mass Amicon ultrafiltr. In the absence of direct Salmonella-epithelial cell contact, release of PEEC bioactivity into the apical reservoir does not occur (+, p < 0.001; **, p < 0.01). Data are expressed as the mean and SD of triplicate samples. One of at least three experiments performed with similar results is shown.
that occurs at the monolayer surface once nonattached bacteria are washed away (as is the standard for the assay). Thus, utilizing such bacterial-soluble products under conditions that prevented direct \textit{Salmonella}-epithelial contact did not result in spontaneous PMN transepithelial migration (0.255 ± 0.029 and 1.16 ± 0.06 CE × 10^4 vs 0.432 ± 0.20 for 5 × 10^6 and 5 × 10^7 bacteria/ml vs negative control, NS). In addition, attempts to isolate PEEC from cultured \textit{S. typhimurium} and compared with supernatants collected from the apical compartment (20×) concentrated for their ability to induce neutrophil transepithelial migration. Only supernatants collected from the apical compartment of \textit{Salmonella}-colonized monolayers induced neutrophil transepithelial migration. F corresponds to filtrate and represents the material that was passed through a specific m.w. cut-off filter; R corresponds to retentate and represents the material that is retained above a specific m.w. cut-off filter. Neat refers to unmanipulated original collected supernatants. Data are expressed as the mean and SD of triplicate samples. One of at least three experiments performed with similar results is shown.

**Crude physical characterization of PEEC bioactivity**

As shown in Figure 5A, PEEC bioactivity passes through a 3000 nominal m.w. cut-off filter, but is retained above a 1000-nominal-m.w. cut-off filter, strongly suggesting that the m.w. characteristics of this soluble chemoattractant are between 1000 and 3000 Da. That measurable amounts of bioactivity were not found in the...
3000-m.w. cut-off retentate was not surprising given that neat apical supernatants similarly did not elicit significant bioactivity and, additionally, greater than 95% of the sample with m.w.s less than 3000 were passed through this filter and concentrated in the 1000-m.w. cut-off retentate. Thus, only concentrated sample from the 1000-m.w. cut-off retentate elicited the ability to induce neutrophil transepithelial migration. Additionally, subsequent dilutional analysis of such apical supernatants, as shown in Figure 5, failed to show a promotion of chemotactic activity in the transepithelial migration assay, indicating that the concentration of PEEC isolated (20× concentrated by volume) did not induce tachyphylaxis. Moreover, the concentration of PEEC isolated by this procedure is sufficiently low enough and pure enough that, according to a 280-UV scan, no material was detected. Importantly, these filtration characteristics also permit semipurified PEEC activity to be isolated and thus concentrated for dose-response studies outlined below and, additionally, permit buffer exchange and washing of PEEC bioactivity. Subsequent experiments use this semipurified form of PEEC concentrated 20× unless otherwise indicated.

To better define the polarity of PEEC release, ultrafiltration studies isolating 20× concentrated basolateral supernatants (in the 1000–3000 nominal m.w. range) collected from monolayers apically colonized with S. typhimurium were performed. As demonstrated in Figure 5C, the resultant concentrated basolateral fractions were again ineffective at inducing PMN transepithelial migration. Subsequent dilutional analysis of basolateral supernatants also failed to elicit a chemotactic activity in the transepithelial migration assay (data not shown), indicating that the lack of a chemotactic bioactivity in the 20× concentrated fraction was not explained by tachyphylaxis due to high PEEC concentration. These results indicate that release of PEEC bioactivity is highly apically polarized (at least 20:1), in contrast to the release of chemokines such as IL-8, which are released in a basolaterally polarized fashion.

As shown in Figure 6, PEEC activity is stable to a variety of physical manipulations. It is not significantly attenuated by either 40 μg/ml of trypsin (74 ± 17% of control) or 10 U of protease K (76 ± 19% of control); is not lost after 20 min of boiling (111 ± 19% of control); and withstands vacuum drying (70 ± 15% of control), repeated freeze/thaw cycles (94 ± 13% of control), and shifts in pH ranging from less than 2.0 to greater than 13 (87 ± 10% and 77 ± 7%, respectively). In addition, 85% of the PEEC activity, which is defined by the ability to induce PMN transepithelial migration, could be recovered in the supernatant fraction of ethanol precipitates, while only 16% was recovered from the pellet. PEEC bioactivity, however, was shown to be sensitive to treatment of 95% acetonitrile. Since small peptides can be resistant to proteolysis, and PEEC bioactivity is of low m.w., these features are rather nonspecific in differentiating a PEEC activity due to a polypeptide as opposed to another structure. To further assure that the PEEC activity was not highly hydrophobic, the activity was loaded onto a C-18 (Sep-Pak) column, and activity was assayed in the hexane eluant. Hexane failed to solubilize PEEC bioactivity (data not shown), indicating that PEEC is probably not a lipid. Preliminary absorbance characterization of the PEEC-active fraction (not shown) shows very little detectable material, consistent with a bioactivity that is effective at an exceedingly low concentration.

**PEEC directly signals PMN**

Although PEEC bioactivity displayed PMN chemotactic activity in the transepithelial migration assay, this assay does not differentiate a chemotactic activity that directly signals the PMN as a transepithelial gradient from one that indirectly signals PMN via influencing epithelial signaling cascades. Thus we next determined whether PEEC exerted chemotactic influences by directly signaling to PMN. For this purpose the underagarose chemotaxis assay was employed in which PMN represents the only cell type present. To discern between chemotactic vs chemokinetic responses, a chemotactic index was evaluated for each sample tested (FMLP, PEEC, and HBSS) and compared with the negative (buffer) control.

As shown in Figure 6, PEEC activity is stable to a variety of physical manipulations. It is not significantly attenuated by either 40 μg/ml of trypsin (74 ± 17% of control) or 10 U of protease K (76 ± 19% of control); is not lost after 20 min of boiling (111 ± 19% of control); and withstands vacuum drying (70 ± 15% of control), repeated freeze/thaw cycles (94 ± 13% of control), and shifts in pH ranging from less than 2.0 to greater than 13 (87 ± 10% and 77 ± 7%, respectively). In addition, 85% of the PEEC activity, which is defined by the ability to induce PMN transepithelial migration, could be recovered in the supernatant fraction of ethanol precipitates, while only 16% was recovered from the pellet. PEEC bioactivity, however, was shown to be sensitive to treatment of 95% acetonitrile. Since small peptides can be resistant to proteolysis, and PEEC bioactivity is of low m.w., these features are rather nonspecific in differentiating a PEEC activity due to a polypeptide as opposed to another structure. To further assure that the PEEC activity was not highly hydrophobic, the activity was loaded onto a C-18 (Sep-Pak) column, and activity was assayed in the hexane eluant. Hexane failed to solubilize PEEC bioactivity (data not shown), indicating that PEEC is probably not a lipid. Preliminary absorbance characterization of the PEEC-active fraction (not shown) shows very little detectable material, consistent with a bioactivity that is effective at an exceedingly low concentration.

**FIGURE 6.** PEEC bioactivity is stable. PEEC bioactivity resists pH extremes, freeze/thaw, vacuum drying, boiling, and protease digestion. These features permit further characterization of PEEC activities but, given PEEC’s small nominal m.w., do not strongly favor a particular structural identity (*, p < 0.01).
Table I. The effect of PEEC on neutrophil-directed migration using the underagarose assay

<table>
<thead>
<tr>
<th>Chemotactic index</th>
<th>1 x 10^{-6} M</th>
<th>5 x 10^{-9} M</th>
<th>1 x 10^{-9} M</th>
<th>HBSS^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMLP</td>
<td>2.03 ± 0.23</td>
<td>1.61 ± 0.27</td>
<td>1.12 ± 0.13</td>
<td>0.98 ± 0.25</td>
</tr>
<tr>
<td>PEEC [20-fold]</td>
<td>1:3</td>
<td>1:10</td>
<td>HBSS^+</td>
<td></td>
</tr>
<tr>
<td>Chemotactic index</td>
<td>1.98 ± 0.27</td>
<td>1.40 ± 0.11</td>
<td>1.20 ± 0.17</td>
<td>0.95 ± 0.23</td>
</tr>
</tbody>
</table>

The data are presented as chemotaxis index (mean (n = 5) ± SD) and measured as a ratio of PMN migration to chemoattract/PMN migration to HBSS^+ (buffer in which chemoattractants were suspended). The magnitude of the migration response to either chemoattract or control buffer was assessed as distance migrated in millimeters via an ocular micrometer (see Materials and Methods for details). To discern between chemotaxis vs. chemokinesis, ratios supporting a chemotaxis index of >1 indicate a chemotactic response while chemotactic indices supporting a ratio of <1 indicate a chemokinesis response (21, 22).

Dose response and time course of PEEC release

To compare the phenotype of PEEC-elicited PMN activation with that of PMN activation by other chemokines and agonists, the concentration dependency of PEEC was obtained. For this purpose PEEC was concentrated in HBSS^+ by filtration (200 x volume) and a dilutional assay of PEEC established in the transepithelial migration assay. As shown in Figure 7A, FMLP-elicited transepithelial migration becomes saturated at 10^{-7} M, as previously reported (25). PEEC responsiveness saturated at a 200-fold concentration, and the response range was defined as occurring over a two-log dilution (Figure 7B). Maximal PEEC-elicited PMN transmigration responses ranged from 40 to 85% of maximal FMLP responses (variability depending on donor PMN and age of T84 cell monolayers). In contrast to FMLP-elicited PMN transmigration, the PEEC response exhibited a slightly steeper slope (5.88 vs 7.70 for PEEC vs FMLP) indicative of a more narrow range of concentration over which the response becomes saturated. Thus, these data indicate that maximal PEEC activity can be obtained by a 200-fold concentration step, and such activity once concentrated is comparable to that exhibited by maximal concentrations of a potent model chemokine (FMLP).

We next defined the time course of PEEC release into the apical compartment of T84 monolayers. Following S. typhimurium colonization of T84 monolayer apical surfaces, the PEEC activity was assessed by sequentially collecting apical supernatants at time course intervals of 2, 4, and 6 h (defined in Materials and Methods) and subsequently evaluated for the ability to direct neutrophil transepithelial migration to imposed gradients. We found that PEEC release (concentrated 20x by volume) into the apical supernatant occurs over a relatively short time course such that by 2 h, >90% of the PEEC activity is released into the apical compartment. Although analyses indicated that the PEEC activity peaked after 4 h, such activity only exhibited a modest increase over that initially secreted in the first 2 h. By 6 h there was a 42% reduction in PEEC activity that was released into the apical compartment of T84 monolayers (11.15 ± 1.24, 12.42 ± 1.72, 7.75 ± 2.00 vs 0.154 ± 0.53 CE 1 x 10^4 for PEEC collected after 2, 4, and 6 h vs the negative HBSS^+ control, respectively). We do note, however, a consistent but statistically insignificant difference between these time points. Thus, release of PEEC into the apical compartment of T84 cell monolayers occurs as an early response to surface colonization.
Apical PEEC secretion in response to apical Salmonella colonization is dependent on protein synthesis by epithelial cells

We next sought to determine whether *S. typhimurium* or epithelial cells were responsible for the secretion of PEEC into the apical epithelial compartment by exploiting selective inhibitors of eukaryotic (CHX) vs prokaryotic (CAM) protein synthesis. An initially complicating feature of these studies was the fact that after pathogen-epithelial apical contact, sequential series of new genes are transcribed to permit the evolving pathogen-eukaryote relationship. However, we have previously shown that pathogen-epithelial cell interrelationships required for induction of epithelial proinflammatory responses are fulfilled within ~45 min of epithelial-pathogen contact (4, 6). We show here (see above) that PEEC secretion is not limited to a narrow time window and occurs continuously even after the first hour of colonization. Thus, since bacterial-epithelial contact is required for eliciting proinflammatory responses, we were able to permit pathogen-epithelial relationships to evolve (45 min), treat them with selective protein synthesis inhibitors (1 h), wash the monolayers, and then collect apical supernatants to be analyzed for PEEC bioactivity. As shown in Figure 8, treatment of *S. typhimurium*-colonized T84 monolayers with the eukaryotic protein synthesis inhibitor CHX completely inhibited apical secretion of PEEC-like bioactivity (*p < 0.001*). Under these conditions of CHX treatment, sensitive indicators of epithelial monolayer function (resistance and Cl secretory responses to agonists) were unaffected (not shown) (6). In contrast, following of *S. typhimurium*-colonized T84 cell monolayers with CAM at concentrations sufficient to selectively inhibit bacterial protein synthesis (24), substantial PEEC activity continued to be secreted (no statistical difference between the CAM vs the no treatment group). These data indicate that, following established pathogen-epithelial interactions, eukaryotic protein synthesis is required for PEEC secretion and suggest that PEEC bioactivity is epithelially derived.

**PEEC signaling to PMN**

A wide variety of neutrophil chemoattractants utilize signal transduction pathways that route through a GTP-binding protein exhibiting the pertussis toxin-sensitive G<sub>αi</sub> subunit (26). Pertussis toxin-mediated ADP ribosylation of this α subunit effectively uncouples signal transduction mediated by G<sub>αi</sub> containing heterotrimeric GTP-binding proteins. As shown in Figure 9A, PMN treatment with either 2 μg/ml of pertussis toxin or vehicle alone (see Materials and Methods) were subsequently examined in the transepithelial transmigration assay for responses to imposed gradients of PEEC or IL-8 (500 ng/ml, the later serving as a positive control for pertussis toxin effects). As shown, pertussis toxin diminished the PMN chemotactic response to both IL-8 (~40%) and PEEC (~78%), indicating that PEEC shares this common signal transduction pathway utilized by several families of PMN chemoattractants that influence PMN movement via G protein-coupled surface receptors.
Known PMN chemoattractants that signal via several distinct surface receptors also appear to share Ca\textsuperscript{2+} mobilization as a potential second messenger. Thus, we compared the ability of PEEC to the known potent chemoattractants, FMLP and IL-8, which have receptors distinctive from one another and also from that utilized by PEEC (see above), to elicit a rise in PMN cytosolic calcium ([Ca\textsuperscript{2+}]\textsubscript{i}). As shown in Figure 9B, PEEC and IL-8 both stimulated rapid and transient increases (ranging from 200–370 nM) in intracellular free calcium ([Ca\textsuperscript{2+}]\textsubscript{i}). All responses reached a maximum within approximately 10 s of stimulation and returned to basal levels by 5 min.

While many of the chemokines share G\textsubscript{q} and intracellular Ca\textsuperscript{2+} signal transduction pathways, they also influence global PMN responses in a distinctive fashion and thus produce activation patterns that further distinguish their unique nature. Thus, we next sought to determine whether PEEC could elicit superoxide production and/or release of primary and secondary PMN granules, and compared the phenotype of this response to that elicited by other chemokines or agonists that activate PMN. These studies were performed at saturating concentration of agonist, and PMN responses were assessed in both the presence or absence of dihydrocytochalasin B (the latter condition amplifies PMN superoxide production and degranulation responses (27)). As shown in Table II, PEEC was similar to IL-8 in its failure to stimulate superoxide production under any condition. However, FMLP, like PMA, stimulated superoxide production that was enhanced by the addition of dihydrocytochalasin B. Moreover, PEEC also did not stimulate either primary (myeloperoxidase) or secondary granule (lactoferrin) release; in fact, PEEC may show a down-regulation of this response. In contrast, FMLP, and to a somewhat lesser extent, LTB4, elicited degranulation of both primary and secondary granules that was further enhanced by the addition of dihydrocytochalasin B. IL-8, in contrast, elicited degranulation of primary granules but not secondary granules. The data describing the distinctive phenotypes of the IL-8, PMA, LTB4, and FMLP responses are consistent with that reported by others (28, 29, 26).

### Discussion

Many inflammatory diseases involving surfaces lined by polarized epithelia are characterized by transepithelial migration of PMN, an event that correlates with epithelial dysfunction and thus clinical symptoms. It is now clear that epithelial cells themselves may emit signals that regulate neutrophil movement and responses at such surfaces. Nowhere is evidence for such epithelial contribution to inflammatory responses more clear than in pathogen-epithelial interactions (4, 6–8, 30, 31). To this end, we have recently shown (4, 6) that biophysically confluent model intestinal epithelial monolayers, when apically colonized by *S. typhimurium*, secrete signals, largely representing basolaterally secreted IL-8, that impart chemotactic memory on underlying matrices. In this fashion, basolaterally secreted signals from epithelia may thus play a role in supporting PMN migration through the underlying matrix by positioning PMN in the immediate subepithelial space. Such secretion, however, does not appear to explain the final step of transepithelial migration of PMN, given that not only would gradients of basolaterally secreted chemotactic activities be reversed from those that would likely induce directed transepithelial migration, but also neutralization of the basolaterally secreted IL-8, as shown here, does not influence this final step of transepithelial migration of PMN.

Such observations led us to speculate that, in addition to basolaterally secreted regulators of the acute inflammatory response, other epithelial signal(s) perhaps including apically secreted mediators may assist in the movement of PMN across the epithelium and into the lumenal compartment. In this study we now report the identification and initial characterization of a novel soluble bioactivity that is important for regulating this later step of transepithelial PMN migration. We demonstrate that model human intestinal epithelial cells, when apically colonized with *S. typhimurium*, release PEEC bioactivity that has properties of a PMN chemokine and is secreted in a polarized apical manner.

The PEEC bioactivity harbors properties that appear to set it apart from the other known peptide- or lipid-based neutrophil chemoattractants. For instance, ultrafiltration results indicate that PEEC exhibits a nominal molecular mass in the range of 1000 to 3000 Da, and behaves in a fashion inconsistent for C5a (≈16,000 m.w.; 32), C-C, or C-X-C family member cytokines (>7000 m.w.;

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**Table II. Effect of PEEC on neutrophil O\textsubscript{2-} production and degranulation**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total O\textsubscript{2-} (nmol/10\textsuperscript{6} PMN)</th>
<th>V\textsubscript{max} O\textsubscript{2-} (nmol/min 10\textsuperscript{6} PMN)</th>
<th>1° Granule Release Myeloperoxidase (OD units)</th>
<th>2° Granule Release Lactoferrin (OD units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMLF</td>
<td>64.8</td>
<td>14.9</td>
<td>0.121 ± 0.008</td>
<td>1.1 ± 0.15</td>
</tr>
<tr>
<td>FMLF + dHCB</td>
<td>352</td>
<td>51.2</td>
<td>0.615 ± 0.02</td>
<td>2.1 ± 0.041</td>
</tr>
<tr>
<td>PEEC</td>
<td>9.3</td>
<td>1.6</td>
<td>0.047 ± 0</td>
<td>0.09 ± 0.007</td>
</tr>
<tr>
<td>PEEC + dHCB</td>
<td>18.5</td>
<td>0.060 ± 0.001</td>
<td>0.353 ± 0.015</td>
<td>0.01 ± 0.03</td>
</tr>
<tr>
<td>IL-8</td>
<td>0</td>
<td>0.1 ± 0.011</td>
<td>0.05 ± 0.02</td>
<td>0.74 ± 0.07</td>
</tr>
<tr>
<td>IL-8 + dHCB</td>
<td>37</td>
<td>0.26 ± 0.008</td>
<td>1.9 ± 0.25</td>
<td>ND</td>
</tr>
<tr>
<td>LTB4</td>
<td>ND</td>
<td>0.09 ± 0.002</td>
<td>1.6 ± 0.28</td>
<td>ND</td>
</tr>
<tr>
<td>LTB4 + dHCB</td>
<td>ND</td>
<td>0.174 ± 0.007</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PMA</td>
<td>333</td>
<td>45.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HBSS</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HBSS + dHCB</td>
<td>33.3</td>
<td>4.1</td>
<td>1.24 ± 0.014</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Suspensions of 5 × 10\textsuperscript{6}/ml (O\textsubscript{2-} assays) and 3 × 10\textsuperscript{6}/ml (degranulation) PMN were stimulated and the above agonists and responses determined as below. Agonists included FMLF (100 nM), IL-8 (4.5 μg/ml), LTB4 (0.5 μM), and PMA (500 ng/ml). For cytochalasin conditions (+dHCB), cells were preincubated with dihydrocytochalasin B for 4 min (5 μg/ml) prior to addition of agonist.

For O\textsubscript{2-} assays, PMN were suspended in cytochrome c buffer in the presence or absence of superoxide dismutase catalase and stimulated for 10 min. The superoxide dismutase inhibitable reduction of cytochrome c was then determined as described in Materials and Methods.

Lactoferrin was determined by ELISA, and myeloperoxidase (MPO) by enzymatic activity as described in Materials and Methods. Values represent OD after substrate adduction. Each value was determined from 0.2 ml of cells (0.6 × 10\textsuperscript{6} PMN); ND, not determined. Data are for one of two experiments.
Although subsets of bacterially derived n-formyl peptides could well be retained in the 1- to 3-kDa fraction, as is PEEC bioactivity, n-formyl peptides do not appear to account for the PEEC bioactivities observed. Given the numbers of bacteria present in the assay (3 x 10^9), known bacterial masses (10^-14 g/microgram), and the known apical bath volume (1 ml), one can calculate whether n-formyl peptide-based signaling could possibly account for the transepithelial migration seen. Such analyses indicate that even if the entire bacterial mass present in the assay consisted of the potent, small m.w., n-formyl peptide, FMLP, the gradient across the monolayer would be insufficient to induce transepithelial migration (6, 25). Thus the observed transmigration would not be expected even if the entire dry weight of the bacteria present represented small, potent n-formyl peptides. Perhaps more importantly, inhibition of the PMN n-formyl peptide receptor does not influence PEEC responses, and PEEC-based patterns of PMN activation do not correspond to those elicited by activation of n-formyl peptide receptors.

PEEC bioactivity also appears to be derived from epithelia since it is dependent on continuous protein synthesis by epithelial cells (and not by bacteria), only after establishment of bacterial-epithelial contact. The above argument, derived from consideration of bacterial mass in the assay and apical volumes in which the assays are employed, also supports the explanation that PEEC is derived from epithelia. That is, given the likelihood that PEEC (like other chemokines) will signal in the nM to mM range, and considering the bacterial mass present, luminal reservoir size of 100 micro liter, and size of PEEC, there is insufficient bacterial product to mount such a response.

The physical characteristics of PEEC activity, however, do not clearly identify its nature, given that PEEC readily signals PMN in buffer solution, is not hexane soluble, and can be easily reconstituted in buffer subsequent to drying on glass in a rotary evaporation device. Thus, it appears that PEEC exhibits at least moderate hydrophilicity. The simplest interpretation from the protein synthesis inhibition data is that PEEC is a peptide, although clearly other interpretations are possible (i.e., a nonpeptide structure that depends on induction of an enzyme for its synthesis). Bulk purification of PEEC for more detailed structural analyses has been confounded by the observation that epithelial cells must be highly polarized, and therefore grown on permeable supports. However, if it is a polypeptide, PEEC resistance to proteolysis and acid degradation is not surprising given its small size. Acid hydrolysis, a general approach in protein degradation, has been also applied to complex homogenates to isolate small, bioactive peptides such as defensins, guanylin, and vasoactive intestinal peptide (37–39). However, the ability of IL-8 to produce a respiratory burst is controversial and our data conflict with that of Baggioni, Imboden, and Detmers, who report that a respiratory burst can be detected when preparations of human neutrophils that respond to FMLP are stimulated with IL-8 (43). Additionally, FMLP, when compared with IL-8, was much more potent in its ability to elicit the release of primary granules from neutrophils and likewise strongly stimulated degranulation of secondary granules and induced neutrophil respiratory burst. Similarly, the preferential release of secondary granules as opposed to primary granules contrasts the strongly chemotactic lipid mediator LTb4 to other chemokines (29). PEEC activity also has a unique profile in terms of global PMN activation, and of the chemokines examined has characteristics more like IL-8. Interestingly, while PEEC exhibits maximal chemotactic activity in the same range as the potent chemotactic peptide FMLP, in contrast to FMLP and other strong PMN chemokines, PEEC has either no detectable or a minimal effect on respiratory burst or degranulation of either primary or secondary granules. Since a PEEC dose-response curve was established by concentration of the PEEC bioactivity, and this activator was compared with all others at identical effective (i.e., saturating) concentrations, such results indicate a unique aspect of PEEC as a nearly “pure” chemoattractant with regard to activation of PMN. Members of the functionally defined family of pure chemoattractants include TGF-β and substance P, and are characterized by the inability to provoke cell-cell aggregation, secretion of O2, or lysosomal enzymes at any concentration (44, 45). In contrast to PEEC bioactivity, these previously described chemoattractants activate G proteins without eliciting increments in cytosolic Ca^2+ (44, 45). In addition, the molecular mass of TGF-β (M, 25,000) is another defining characteristic that separates PEEC from TGF-β (44, 45). Thus, taken together, PEEC appears to exhibit characteristics that are distinct even from known “pure” neutrophil chemoattractants.

In summary, apical attachment of Salmonella to epithelial monolayers induces a host of epithelial responses that serve to coordinate the recruitment of neutrophils to the luminal space in which the pathogen is positioned. Although not involved in transepithelial migration, polarized basolateral release of IL-8 can serve, as we have previously shown (4), to imprint subepithelial matrices with long-lived chemotactic gradients that resist the sweeping-away effects of large-scale water transport that characterize transporting mucosal surfaces. Thus, in the final step of transepithelial migration, subepithelial PMN would be ideally positioned to traffic across the monolayer in response to secreted PEEC activity. Such a presumption has recently been corroborated by the work of Rothenberg et al. (46), who demonstrate that intranasal or s.c. application of eotaxin selectively recruits large numbers of eosinophils into the mouse lung and skin, respectively. Therefore, given that apically directed PMN migration is by itself thought to contribute to epithelial dysfunction in a host of mucosal diseases (1–3, 47–51), it is possible that interference with the PEEC-based signaling pathway may provide a potentially important new therapeutic target for treatment of acute inflammatory diseases of intestinal and perhaps other mucosal surfaces.

References


