Salmonella typhimurium Attachment to Human Intestinal Epithelial Monolayers: Transcellular Signalling to Subepithelial Neutrophils

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Abstract. In human intestinal disease induced by *Salmonella typhimurium*, transepithelial migration of neutrophils (PMN) rapidly follows attachment of the bacteria to the epithelial apical membrane. In this report, we model these interactions in vitro, using polarized monolayers of the human intestinal epithelial cell, T84, isolated human PMN, and *S. typhimurium*. We show that *Salmonella* attachment to T84 cell apical membranes did not alter monolayer integrity as assessed by transepithelial resistance and measurements of ion transport. However, when human neutrophils were subsequently placed on the basolateral surface of monolayers apically colonized by *Salmonella*, physiologically directed transepithelial PMN migration ensued. In contrast, attachment of a non-pathogenic *Escherichia coli* strain to the apical membrane of epithelial cells at comparable densities failed to stimulate a directed PMN transepithelial migration. Use of the n-formyl-peptide receptor antagonist *N*-t-BOC-l-methionyl-l-leucyl-l-phenylalanine (tBOC-MLP) indicated that the *Salmonella*-induced PMN transepithelial migration response was not attributable to the classical pathway by which bacteria induce directed migration of PMN. Moreover, the PMN transmigration response required *Salmonella* adhesion to the epithelial apical membrane and subsequent reciprocal protein synthesis in both bacteria and epithelial cells. Among the events stimulated by this interaction was the epithelial synthesis and polarized release of the potent PMN chemotactic peptide interleukin-8 (IL-8). However, IL-8 neutralization, transfer, and induction experiments indicated that this cytokine was not responsible for the elicited PMN transmigration. These data indicate that a novel transcellular pathway exists in which subepithelial PMN respond to lumenal pathogens across a functionally intact epithelium. Based on the known unique characteristics of the intestinal mucosa, we speculate that IL-8 may act in concert with an as yet unidentified transcellular chemotactic factor(s) (TCF) which directs PMN migration across the intestinal epithelium.

ATTACHMENT of an array of bacterial pathogens to epithelial surfaces is accompanied by recruitment of host defense cells as manifested by transepithelial migration of neutrophils (PMN)[1]. For example, PMN migration across renal tubular, urinary transitional, or bronchial epithelia represents the histological definition of acute bacterial pyelonephritis, acute cystitis, and acute bronchitis, respectively (Ivanyi et al., 1983; Koyama et al., 1991; Tsujimura et al., 1980). Similarly, transmigration of PMN across intestinal epithelia is a hallmark of bacterial enterocolitis, exemplified by salmonellosis (Day et al., 1978; Kumar et al., 1982; Yardley and Donowitz, 1977; McGovern and Slavutin, 1979; Rout et al., 1974; Takeuchi, 1967).

Salmonella typhimurium is the most common *Salmonella* serotype isolated from humans suffering from infectious gastroenteritis and correspondingly has long been recognized as a public health problem. Contact between *Salmonella* and epithelial cell apical membranes elicits a variety of epithelial responses. For example, the architecture of the epithelial cytoskeleton underlying the contact site is dramatically reordered, as are patterns of epithelial protein phosphorylation (Finlay and Falkow, 1990; Galan et al., 1992*a*,*b*; Takeuchi, 1967). Such epithelial responses are likely triggered by specific contact-dependent, bacterial-derived signals which are themselves modulated by physical characteristics of the microenvironment such as oxygen tension and osmolarity

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1. *Abbreviations used in this paper*: Ab, antibody; CE, cell equivalents; CFU, colony forming units; PMN, polymorphonuclear leukocyte; tBOC-fMLP, *N*-t-BOC-l-methionyl-l-leucyl-l-phenylalanine; TNF, tumor necrosis factor; UTIs, urinary tract infections.

(Ernst et al., 1990; Galan and Curtiss, 1990; Lee and Falkow, 1990; Scheimann and Shope, 1991). Subsequent to such alterations, *Salmonella* may be internalized in a membrane bound vacuole and may translocate across the intestinal epithelium (Finlay et al., 1988).

The details of how such *Salmonella*-intestinal epithelial contacts evoke the classical histological lesion of PMN transepithelial migration are not known (McGovern and Slavutin, 1979; Rout et al., 1984; Takeuchi, 1967). However, it is clear that transepithelial migration of PMN occurs early after *Salmonella*-epithelial contact (Takeuchi, 1967) and well before the epithelium loses its structural integrity (Day et al., 1978). Such observations imply that contact between the bacterial outer membrane and the epithelial apical membrane results in the generation of a signal which directs subsequent trafficking of PMN.

Unraveling the relationships between *Salmonella* attachment to intestinal epithelia, epithelial barrier function, and the subsequent effects on neutrophil epithelial interactions require a reductionistic model system. We have previously modeled intestinal epithelial-PMN interactions using human peripheral blood neutrophils in conjunction with monolayers of the human-derived, columnar, physiologically confluent, crypt-like intestinal epithelial cell line, T84 (Colgan et al., 1993; Madara et al., 1993; Nash et al., 1987; Parkos et al., 1991, 1992). We now extend this system to examine how *Salmonella*-intestinal epithelial interactions modulate subsequent PMN-epithelial interactions. Here we show that apical attachment of *S. typhimurium* to intestinal epithelial monolayers has a profound stimulatory effect on subsequent physiologically directed PMN transepithelial migration. This pathogen-induced PMN transmigration does not depend on the n-formylated peptide signaling mechanism, which classically directs PMN to bacteria and occurs under conditions in which epithelial barrier function is maintained. The chemotactic signal generated requires bacterial-apical membrane contact and subsequent intact protein synthesis in both the bacterium and the intestinal epithelial cell. Although we show such interactions result in the polarized secretion of the potent chemotactic peptide IL-8 from the epithelial cell, IL-8 secretion fails to explain the observed transepithelial migration of PMN in response to apical colonization by *Salmonella*. Such data suggest that a novel mammalian defensive mechanism has evolved in which epithelial cells can signal and recruit underlying inflammatory cells in response to pathogens which adhere to their apical membrane. This strategy may be used to mobilize PMN and allow for their directed transepithelial migration before injury to the epithelial surface has occurred.

Materials and Methods

Approximately 700 monolayers were used for these studies.

Cell Culture

T84 intestinal epithelial cells (passages 70–95) were grown and maintained as confluent monolayers on collagen-coated permeable supports (Dharmsathaphorn and Madara, 1990) with recently detailed modifications (Madara et al., 1992). Briefly, monolayers were grown on 0.33 cm^2 ring-supported polycarbonate filters (Costar Corp., Cambridge, MA) and used 6–14 d after plating (Madara et al., 1992). Inverted monolayers, used to study transmigration of PMN in the physiological basolateral-to-apical direction were constructed as described before (Madara et al., 1992; Nash et al., 1991; Parkos et al., 1991).

Bacterial Strains and Growth Conditions

Bacterial Strains. Amer. Type Culture Collection (Rockville, MD) strain 14028, a fully virulent strain of *S. typhimurium*, is the wild-type parent strain used in these studies. *E. coli* F-18, isolated from the feces of a healthy human in 1977, is a streptomycin-resistant (Str^r) and rifampin-resistant (Rif^r) derivative of *E. coli* F-18 and is an excellent colonizer of the streptomycin-treated mouse large intestine (Cohen et al., 1983; Myhal et al., 1982). The strain produces type 1 pili and colicin V, and its serotype is rough:K1:H5 (McCormick et al., 1989).

Media. Luria broth (LB) was made as described by Revel (1966). L agar is L broth containing 12 g of Bacto Agar (Difco Laboratories Inc., Detroit, MI) per liter. MacConkey agar (Difco Laboratories Inc.) was prepared according to package instructions.

Growth Conditions. Non-agitated microaerophilic cultures were prepared by inoculating 10 ml of LB broth with 0.01 ml of a stationary-phase culture, followed by overnight incubation (~18 h) at 37°C. Bacteria from such cultures were in the late logarithmic phase of growth and correlated with 5–7 × 10^8 colony forming units (CFU)/ml, routinely. CFU were determined by diluting and plating onto MacConkey agar medium (Difco Laboratories Inc.) or L agar, accordingly.

S. typhimurium Invasion into T84 Intestinal Epithelial Monolayers

Infection of T84 monolayers was performed by the method of Lee and Falkow (1990) with slight modification. Inserts with attached monolayers were lifted from wells, drained of media by inverting, and gently washed by immersion in a beaker containing HBSS(+) (containing Ca^{2+} and Mg^{2+}, with 10 mM Hepes, pH 7.4, Sigma Chem. Co., St. Louis, MO). The monolayers were placed in a new 24-well tissue culture plate with 1.0 ml HBSS(+) in the lower (outer) well and 0.05 ml HBSS(+) added to the upper (inner) well. After a 30–45-min equilibration, 7 μl of each (HBSS(+) washed) bacterial sample (representing an inoculation ratio of 10–20 bacteria/epithelial cell) were added per monolayer, and bacterial invasion was followed throughout a time course of 1 h. Cell associated bacteria represent populations of bacteria adherent to and/or internalized into the T84 monolayers and are released by incubation with 0.1 ml of 1% Triton X-100 (Sigma Chem. Co.). Internalized bacteria were those obtained from lysis of the epithelial cells with 1% Triton X-100, 20 min after the addition of gentamicin (50 μg/ml). Gentamicin, an aminoglycoside antibiotic, does not permeate eukaryotic plasma membranes and is therefore cytolytic only to extracellular populations of bacteria while intracellular bacteria populations remain viable (Lissner et al., 1983). Preliminary gentamicin dose-response studies defined the conditions required to achieve bacteriocidal effects on the strain used (data not shown). For both cell associated and internalized bacteria, 0.9 ml LB broth was then added and each sample was vigorously mixed and quantitated by plating for CFU on MacConkey agar medium.

Electron Micrographs of S. typhimurium Interacting with T84 Cell Polarized Monolayers

After incubation for 15, 45, or 120 min with *S. typhimurium*, T84 inserts were fixed at room temperature for 1 h in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4. Inserts were then washed with 0.1 M Na cacodylate buffer, postfixed for 1 h at 4°C with 1% osmium tetroxide in 0.1 M Na cacodylate buffer, en bloc stained with uranyl acetate, and washed and dehydrated in a graded series of ethanols. Inserts were embedded in LX-112 resin and polymerized overnight in a 60°C oven. Sections of 0.5 μm were obtained and stained with toluidine blue. Thin sections of ~60 nm were cut, stained, and subsequently examined and photographed using a Phillips 201 electron microscope.

Electrical Measurements

To assess currents, transepithelial potentials, and resistance, a commercial voltage clamp (Bioengineering Department, University of Iowa) was employed and interfaced with an equilibrated pair of calomel electrodes submerged in saturated KCl along with a pair of Ag-AgCl submerged in HBSS(+). Agar bridges were used to interface the electrode with the solutions on either side of the monolayers (one calomel and one Ag-AgCl electrode in each well) and measurements of short circuit current and resistance were made as detailed elsewhere (Madara et al., 1992).

PMN Transepithelial Migration Assay

The physiologically directed (basolateral-to-apical) PMN transepithelial migration assay has been previously detailed (Parkos et al., 1991; Madara et al., 1992). Human PMN were isolated and suspended as described elsewhere (Parkos et al., 1991) at a concentration of 5×10^7/ml. Before addition of PMN, T84 inverted monolayers were extensively rinsed in HBSS(+) to remove residual serum components. *S. typhimurium* were prepared by washing twice in HBSS(+) and were resuspended at a final concentration of $\sim 5 \times 10^9$/ml. 100 μl bacterial aliquots ($\sim 5 \times 10^8$) were microcentrifuged at 14 kx*g* for 3 min and resuspended in a final volume of 25 μl. Inverted monolayers were removed from each well and placed in a moist chamber such that the epithelial apical membrane was oriented upward. The bacterial suspension was gently distributed onto the apical surface and incubated for 45 min at 37°C. Non-adherent bacteria were removed by washing three times in HBSS(+) buffer. The monolayers were then transferred, by inverting, back into the 24-well tissue culture tray containing 1.0 ml HBSS buffer in the lower (apical membrane now colonized with *Salmonella*) reservoir and 160 μl in the upper (basolateral interface) reservoir. For simplicity, the reservoir will be referred to according to which epithelial membrane domain they interface with (i.e., apical or basolateral). To the basolateral bath, 40 μl (10^6) of isolated PMN were added to each monolayer and incubated for 110 min at 37°C. Positive control transmigration assays were performed by addition of chemoattractant (1 μM fMLP) to the opposing apical reservoir. All experiments were performed in a 37°C room.

Transmigration was quantitated by a standardized assay for the PMN azurophilic granule marker myeloperoxidase (Parkos et al., 1991, 1992). After each transmigration assay, non-adherent PMN were extensively washed from the surface of the monolayer and PMN cell equivalents, estimated from a standard curve, were assessed as the number of PMN associated with the monolayer, and the number that had completely traversed the monolayer (i.e., into the apical reservoir).

Gentamicin and Chloramphenicol Treatment of S. typhimurium

After the 45 min colonization step as described above, T84 cell-associated *S. typhimurium* were treated with gentamicin (50 μg/ml) for 20 min, as described above, to eliminate the extracellular (or adherent) populations of bacteria. Monolayers were extensively washed (>five times) in HBSS(+) buffer to remove residual gentamicin, placed back into the 24-well tray, and subsequently assayed for PMN transmigration as detailed above. In the subsets of experiments indicated, chloramphenicol (100 μg/ml), a bacteriostatic protein inhibitor (which does not similarly affect eukaryotic cells) was added to *S. typhimurium* (MacBeth and Lee, 1993) before the 45 min colonization step and remained in the apical reservoir throughout the course of PMN transmigration.

Cycloheximide Treatment of T84 Monolayers

Cycloheximide (Sigma Chem. Co.), an inhibitor of eukaryotic but not prokaryotic protein synthesis was prepared as a 2 mg/ml stock in 95% ethanol and diluted to 2 μg/ml in media. This concentration has been found to inhibit 75% of radiolabeled leucine incorporation into T84 cell precipitable protein (McRoberts et al., 1990).

N-t-BOC-l-methionyl-l-leucyl-l-phenylalanine Pretreatment of PMN

N-t-BOC-l-methionyl-l-leucyl-l-phenylalanine (tBOC-fMLP), an n-formyl peptide receptor antagonist, was purchased from Sigma Chem. Co. PMN (2.5×10^7/ml) were preincubated in the presence or absence of 300 μM tBOC-fMLP for 5–10 min at 37°C. Subsequently, PMN (40 μl or 10^6) were added to the basolateral (upper) reservoir of T84 cell inverted monolayers treated under one of the following conditions: (*a*) addition of chemoattractant (1–100 nM fMLP) to the apical (lower) reservoir, (*b*) *S. typhimurium* apical colonization of T84 monolayers, as described above, or (*c*) addition of bacterial supernatants to the apical reservoir (see below).

Preparation of S. typhimurium Cultured Supernatants

Overnight cultures of microaerophilically grown *S. typhimurium* were washed twice in HBSS(+) buffer and resuspended to a final concentration of $\sim 5 \times 10^7$/ml. After an incubation for 1 h at 37°C, the suspensions were spun free of bacteria (14K, 3 min) and the supernatant (termed *Salmonella* conditioned buffer supernatant) was collected, passed through a 0.2-μm filter, and stored at −70°C.

Detection of IL-8 Secreted by T84 Epithelial Cells Stimulated with S. typhimurium

Microaerophilic cultures of *S. typhimurium* were washed twice in HBSS(+) buffer and resuspended at a final concentration of 5×10^9/ml. 100-μl aliquots were added to the apical surface of inverted monolayers as described above for PMN transmigration. After 45 min the non-adherent bacteria were removed by gently washing with HBSS(+) buffer and fresh buffer was added such that 140 μl and 1 ml were added to the basolateral and the apical aspect of the monolayer, respectively. After a 2-h incubation at 37°C, the supernatants from both sides of the monolayer were collected and assayed for IL-8 using slight modifications of a previously reported method (Schurer-Maly et al., 1992). The level of IL-8 was determined by an ELISA using polyclonal rabbit anti-IL-8 antibody (Endogen, Inc., Boston, MA) as a capturing antibody (Ab) and a polyclonal goat anti-IL-8 Ab (R and D Systems) as a detection Ab. Peroxidase conjugated rabbit anti-goat IgG (Sigma Chem. Co.) was used as the reporting antibody. After an addition of the substrate, ABTS, plates were read at a wavelength of 405 nm.

IL-8 Neutralization Studies

We used a neutralizing anti-IL-8 Ab (Endogen Inc.) to determine the effects of IL-8 in this model system for PMN transepithelial migration. 30 μg/ml anti-IL-8 was added to both sides of T84 monolayers either subsequent to *S. typhimurium* colonization or before adding imposed gradients of fMLP or IL-8 (100 ng/ml). 10^6 PMN/monolayer were then added to the basolateral reservoir and after 120 min were assessed for their ability to migrate transepithelially via an enzymatic assay for the PMN specific marker myeloperoxidase (Parkos et al., 1991, 1992).

Data Presentation

Since variations exist in both transepithelial resistance between groups of monolayers (baseline resistance range 650–1,500 ohm $\times$ cm^2) and in PMN obtained from different donors, individual experiments were performed using large numbers of monolayers and PMN from single blood donors on individual days. PMN isolation was restricted to 10 different donors (repetitive donations) over the course of these studies. *Salmonella* invasion, and myeloperoxidase assay data were compared by Student's *t* test. PMN transmigration results are represented as PMN cell equivalents (CE) derived from a daily standard PMN dilution curve. Monolayer-associated PMN are represented as the number of PMN CE per monolayer and reservoir-associated PMN (i.e., PMN which had completely traversed the monolayer) are represented as the number of PMN CE/ml (total volume 1 ml). Values are expressed as the mean and SD of an individual experiment done in triplicate repeated *n* times.

Results

Characterization of Attachment/Internalization of S. typhimurium by Intestinal Epithelial Monolayers

S. typhimurium was examined for its ability to adhere to and be internalized by polarized T84 intestinal epithelial monolayers throughout a 60-min time course. Initial characterization used an inoculum of 15 bacteria/epithelial cell which was placed on the apical membrane of monolayers grown on permeable supports for periods ranging from 10–60 min (Fig. 1). Increasing durations of exposure led to greater numbers of bacteria which were monolayer associated and internalized (Fig. 1, $p < 0.05$). At any time after epithelial exposure, there were ~20-fold greater bacteria attached than internalized (Fig. 1). For example, after 60 min 5.64 $\pm$ 1.00% of the original *S. typhimurium* inoculum was cell-associated (~50 bacteria/100 epithelial cells) while only 0.25 $\pm$ <0.01% was internalized into T84 cells (<3 bacteria/100 epithelial cells). Although internalization was relatively inefficient, sequential increments in internalization oc-

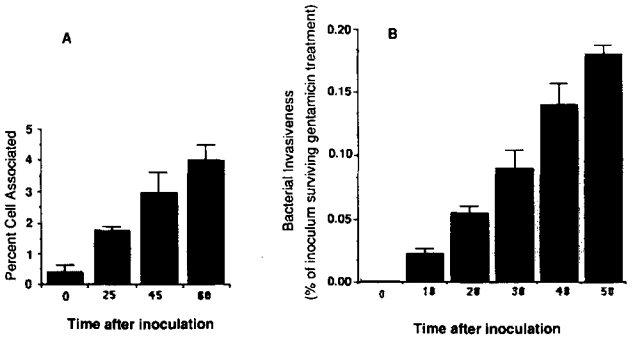

Figure 1. Time dependence of *S. typhimurium* association with (*A*) and invasion of (*B*) T84 monolayers. (*A*) The percent of the initial inoculum which developed cell associations, and (*B*) the percent which became internalized increased with time (association was terminated by vigorous washing followed by a temperature shift to 4°C). Although invasion proceeded as quickly as attachment, the incidence of invasion relative to attachment was low in the first 60 min. Thus, at any given time ~20 bacteria were surface attached for each one internalized.

curring 10 and 20 min after inoculation suggested that the process is rapid (Fig. 1). Dose-response studies showed that increasing the inoculum yielded qualitatively similar time-dependent increments in attachment and internalization and led to enhanced numbers of epithelial-associated bacteria. Indeed, as demonstrated in Fig. 2, increasing inoculation ratios from 15:150:1,500 bacteria/epithelial cell led to progressive increases in cell surface and internalized organisms (0.50 ± 0.28, 15.00 ± 3.40, 80.00 ± 12.00, and 0.03 ± 0.001, 0.20 ± 0.085, 0.48 ± 0.085 bacteria/epithelial cell for attachment and internalization, respectively, all $t = 60$ min). Electron microscopy was used as a means of verifying the nature of *Salmonella*-epithelial associations studied.

As shown in Fig. 3 *c*, *Salmonella* were largely confined to the apical membrane of polarized T84 cells and such bacterial-epithelial associations consisted of close adherence (Fig. 3 *c* [*i*]) which routinely led to architectural reordering of the epithelial surface. For example, *Salmonella* were subsequently internalized via the formation of membrane ruffles into membrane bound vacuoles (Fig. 3 *c* [*ii*], [*iii*], and [*iv*], respectively). However, only rarely were internalized bacteria observed, consistent with the results of the gentimicin-based studies. These structural findings indicate that washes were sufficient to remove unattached bacteria and that bacterial-epithelial adhesive interactions were being appropriately modeled. These data are consistent with previous reports detailing *Salmonella* entry into epithelial cells. Our data, for example, is comparable to electron microscopic studies by Takeuchi (1967) showing the sequence of events leading to bacterial internalization in vivo, and as expected, our data substantiates similar changes which have been observed in vitro when cultured polarized epithelial cells (MDCK and Caco-2) were used (Finlay et al., 1988; Finlay and Falkow, 1990). Also, as depicted in these figures, individual *Salmonella* have diameters of ~0.8–1 μm and thus exhibit individual cross sectional areas of ~0.5–0.8 μm², compared to polarized T84 cells which occupy permeable supports at densities of 10^6 cm² and thus have apical surface areas averaging ~100 μm². Such data indicate that individual epithelial cells could readily accommodate >100 bacteria per epithelial cell.

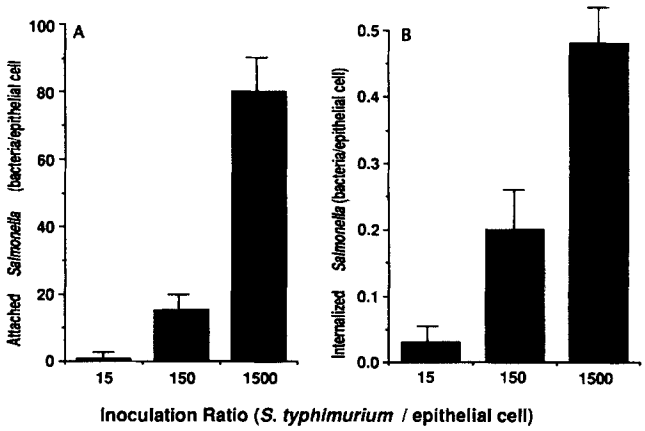

Figure 2. Dose dependence of *S. typhimurium* attachment to (*A*), and internalization (*B*) into T84 monolayers. (*A*) Number of *Salmonella*/epithelial cell which developed cell associations and subsequently (*B*) became internalized, increased with *S. typhimurium* dose (inoculation ratio of 15–1,500 bacteria/epithelial cell), when assayed after 60 min. Cell associated *S. typhimurium* were distinguished from internalized *S. typhimurium* by gentamicin treatment as described in Materials and Methods.

Effect of S. typhimurium Colonization on Epithelial Monolayer Function

Pilot experiments (not shown) indicated that epithelial cell-associated bacterial densities of 80–100, achieved by inoculating 0.33 cm² monolayers with 5×10^8 bacteria for 45–60 min, led to ideally sized signals for the analyses of neutrophil-epithelial interactions described in detail below. Thus, the effects of such colonization densities on monolayer function were determined. Transepithelial resistance to passive ion flow is an extremely sensitive measure of barrier function in high resistance epithelia such as T84 monolayers (Madara, 1990). Indeed, as would be predicted by circuit modeling (Madara and Dharmsathaphorn, 1985), perturbations so minimal that they induce barely detectable increases in transepithelial flux of inert solutes routinely elicit sizable decrements in resistance in such high resistance monolayers (due to the asymptotic flux/resistance relationship) (Hecht et al., 1988; Madara and Dharmsathaphorn, 1985). As demonstrated in Fig. 3 *a*, transepithelial resistance at the 80–100 bacteria/cell colonization density remained high in the 2 h after colonization (598 ± 17 vs 656 ± 35 ohm × cm² for 0 and 120 min postinoculation, respectively, NS). These specific conditions were subsequently used for all of the neutrophil experiments reported below. When postcolonization incubations were extended to 3 h, significant decrements in resistance were obtained (~50% decrease), although physiological confluency was maintained even under these conditions (i.e., resistance values in substantial excess of 250 ohm × cm²) (Hecht et al., 1988; Madara and Dharmsathaphorn, 1985). After 4 h, resistance fell below 250 ohm × cm², but this occurred for both *S. typhimurium* colonized and control noncolonized monolayers. Similarly, in data not shown, we found that loading monolayers with as many as 1,500 *S. typhimurium*/epithelial cell reduced transepithelial resistance significantly (50%) only after 3 h when compared to buffer controls. However, this 50% reduction still gave a transepithelial resistance value of ~400 ohm × cm², representing a substantial barrier function.

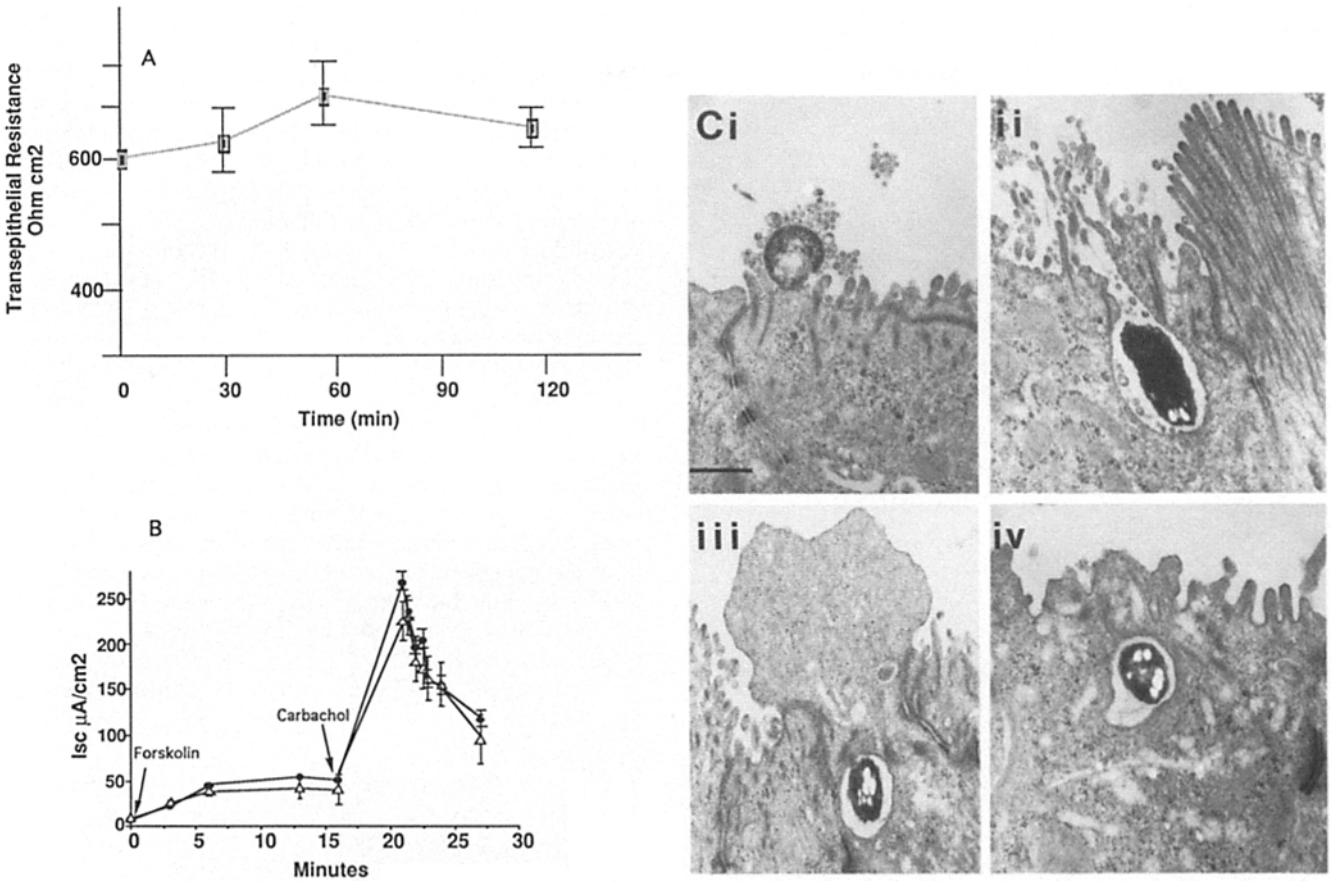

Figure 3. The effects of *S. typhimurium* interactions with polarized T84 monolayers on transepithelial resistance to ion flow (*A*), and ion transport (*B*) and apical membrane structure (*C*). (*A*) Transepithelial resistance to passive ion flow remained high and unchanged even 2 h after establishment of cell-associated *S. typhimurium* colonization at densities of 80–100 bacteria per epithelial cell. The significance of such resistance values has been extensively studied (Madara and Dharmsathaphorn, 1985) and is known to equate with extremely limited permeability to hydrophilic solutes. (*B*) Short circuit currents (*Isc*) induced by the cAMP agonist forskolin (10 μM) and the Ca^{++} agonist carbachol (1 μM) 2 h after monolayer colonization by 80–100 bacteria per cell (*open triangles*) or in control monolayers never exposed to bacteria (*closed circles*). Cell associated bacteria, even at these large numbers, have no effect on the ability of monolayers to respond to forskolin, maintain responses over time, respond to carbachol, and demonstrate potentiated cAMP and Ca^{++} responses. (*C*) Electron micrographs of T84 cell-polarized monolayers infected apically with *S. typhimurium* revealing the anatomical nature of the associations between bacteria and the epithelial cells, represented in this study. (*i*) Individual *Salmonella* closely associate with the apical membrane. *Salmonella* were then subsequently internalized (*ii*) via the formation of membrane ruffles (*iii*) into membrane bound vacuoles (*iv*). These results are comparable to studies reported in natural intestinal epithelial (Takeuchi, 1967), and in vitro polarized epithelial cell model systems (Finlay et al., 1988; Finlay and Falkow, 1990). Bar, ~1 μm.

We next examined if the standard colonization conditions used (80–100 bacteria per epithelial cell) affected the other major functional characteristic of intestinal epithelia–active vectorial ion transport. As their major ion transport pathway, T84 monolayers exhibit electrogenic Cl^- secretion: the transport event underlying hydration of mucosal surfaces and, in the extreme, secretory diarrhea (Donowitz and Welsh, 1987; Madara et al., 1992; Nash et al., 1991). Such Cl^- secretion is regulated in this model similarly to that in natural epithelia (Madara, 1990; Madara and Dharmsathaphorn, 1985), and can be quantitatively assayed by measurement of short circuit currents (Isc) upon stimulation with secretory agonists. Monolayers colonized with *S. typhimurium* under the above conditions were found to normally initiate and sustain responses to the cAMP agonist forskolin (10 μM), normally respond to the Ca^{++} agonist carbachol (1 μM), and exhibit normal Ca^{++}-cAMP potentiated secretory responses (Fig. 3 *b*). Moreover, such responses require the continuous coordinated effort of six membrane transport elements (including two types of Cl channels and K channels) and require continuous upregulated activity of Na-K-ATPase to maintain the electrochemical gradient which underlies such electrogenic Cl^- secretion (Donowitz and Welsh, 1987). Thus, the colonized monolayers to be used for the experiments below maintain both barrier and regulated ion transport characteristics.

Apical Colonization by S. typhimurium Initiates Physiologically Directed Transepithelial Migration of PMN

We next examined whether apical colonization of monolayers by *Salmonella*, under the standard conditions in which the physiological functions of the monolayers are preserved, modulated subsequent neutrophil-epithelial interactions. The maximally effective (10^{-6} M) (Nash et al., 1987) im-

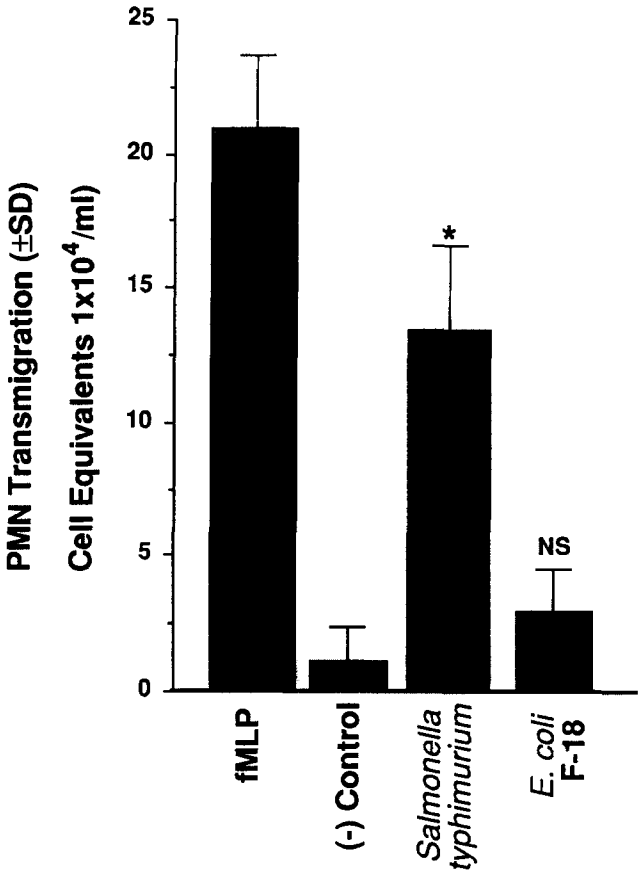

Figure 4. Induction of physiologically directed PMN transepithelial migration (basolateral-to-apical) by apical membrane attachment of *S. typhimurium*. A positive PMN transmigration control was established using imposed maximally effective gradients of the potent chemotactic peptide fMLP. A negative control consisted of no imposed gradient of chemoattractant. In contrast to the negative control, apical colonization of monolayers by *S. typhimurium* elicited >60% of the maximal transmigration response (*$P < 0.005$). *E. coli* F-18, a non-pathogen isolated from the human colon, attached apically at similar densities to *S. typhimurium* (see text) but induced no transepithelially directed chemotaxis of PMN. Data represent the mean and SD of triplicate samples. One out of three experiments performed. NS, not significantly different from negative control.

posed transepithelial gradient of the chemotactic peptide, fMLP, served as a positive control for PMN transmigration (Fig. 4). Apical colonization of T84 monolayers with *S. typhimurium*, by itself, elicited a marked transepithelial migration of PMN in the physiologic basolateral-to-apical direction which was ~60% of that induced by the maximally effective transepithelial gradient of fMLP (Nash et al., 1987) (Fig. 4). In contrast, similar apical exposure to *E. coli* F-18, a normal flora isolate of the large intestine, failed to comparably stimulate detectable PMN transepithelial migration. *E. coli* F-18 was able to attach to T84 polarized monolayers at similar levels as *S. typhimurium*, although no significant internalization was observed (Table I).

The data shown in Fig. 4 represent PMN transmigration. We also measured filter associated PMN, which previous structural studies have shown largely represent PMN which have migrated across the filter but have not yet crossed the tight junction; thus being trapped in the paracellular and subepithelial spaces (Parkos et al., 1991, 1992). The numbers of such monolayer associated PMN were similarly increased in *S. typhimurium* colonized monolayers (2.30 ± 0.60 vs 1.70 ± 0.11 vs 0.39 ± 0.25 vs 3.02 ± 1.66 PMN cell equivalents $\times 10^4$·monolayer/ml, for fMLP, *S. typhimurium*, negative control, and *E. coli* F-18, respectively, $p < 0.05$ for negative control vs *S. typhimurium* colonized). PMN transepithelial migration was dependent on the density of colonization. As shown in Fig. 5, cell-associated *S. typhimurium* at densities of 3, 15, and 80 bacteria/epithelial cell elicited a progressive increase in the number of transmigrating PMN.

Salmonella Induced PMN Transepithelial Migration Occurs via a Non-Classical Mechanism; Transcellular Chemotactic Factor

Since n-formylated peptides are the major bacterial-derived factor known to induce *directed* PMN migration, we next assessed whether the transmigration response required participation of n-formyl peptide receptor-based PMN signaling. As shown in Fig. 6, pretreatment with 300 μM tBOC-MLP, an n-formyl peptide receptor antagonist, inhibited (by 70–90%) the transmigration response of PMN to imposed transepithelial chemotactic gradients of fMLP ranging from 10 to 100 nM. In contrast, 300 μM tBOC-MLP had no effect on the PMN transepithelial migration elicited by surface colonization by *S. typhimurium*. Since, on a molar basis, fMLP is an exceedingly potent activator of n-formyl-peptide receptor mediated chemotaxis (Gallin, 1988), the above results suggest that the PMN transepithelial migration induced by apical colonization with *Salmonella* is not dependent on n-formyl-peptide driven chemotaxis. Further substantiating this notion was the calculation that, given the numbers of colonizing bacteria (3×10^6), bacterial weight (10^{-14} gm/bacteria), apical reservoir volume (1 ml), and n-formyl peptide gradients (Nash et al., 1987) necessary to promote PMN

Table I. Adherence and Internalization of S. typhimurium and E. coli F-18 into T84 Intestinal Epithelial Cell Polarized Monolayers and Effects on PMN Transepithelial Migration‡*

Strain	% adherence	% internalization	PMN transmigration (*CE* 10^4)
S. typhimurium	13.10 ± 2.46	0.34 ± 0.050	13.2 ± 3.00
E. coli F-18	11.90 ± 3.60	0.02 ± 0.004	2.42 ± 2.00

* Values given represent the percent of the original inoculum that was either adherent to or subsequently became internalized (percent *Salmonella* surviving gentamicin treatment) into T84 intestinal epithelial cell polarized monolayers.
‡ Numbers of PMN transmigrated are reported as CE (cell equivalents 10^4), based on a known PMN standard and quantitated using the myeloperoxidase assay. Data represent mean of triplicate samples ± SD. One of at least two experiments.

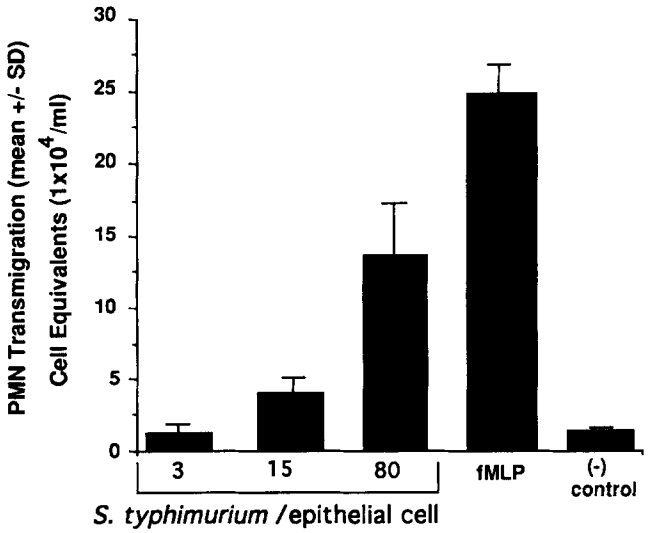

Figure 5. Dose response of *S. typhimurium* induced PMN transepithelial migration. Intestinal epithelial monolayers were colonized apically with 3, 15, or 80 cell associated *S. typhimurium*/epithelial cell. Increasing density of attachment yielded correspondingly greater transepithelial migration. Data are expressed as the mean and SD of triplicate samples for each condition and summarize one out of three experiments performed with comparable results.

transmigration, transmigration such as that observed would not be expected even if the entire dry weight of the bacteria present represented small, potent n-formyl peptides like fMLP.

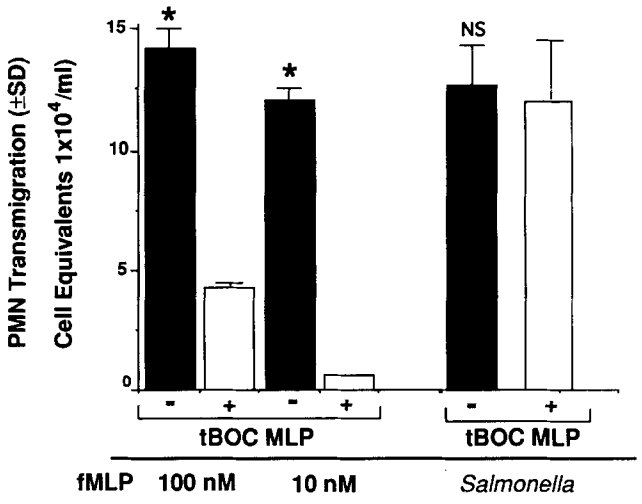

Figure 6. Effect of PMN n-formyl-peptide receptor antagonist t-BOC-l-methionyl-l-leucyl-l-phenylalanine on the ability of *S. typhimurium* to induce PMN transepithelial migration. Intestinal epithelial monolayers were colonized with *S. typhimurium* at a density of 80–100 cell associated bacteria/epithelial cell. PMN with and without preincubation with 300 μM t-BOC-MLP were added to the basolateral aspect of monolayers colonized with *S. typhimurium* or driven to migrate under the influence of imposed fMLP gradients ranging from 10–100 nM. tBOC is effective at inhibiting n-formyl-peptide (*$P < 0.01$ for both gradients) but not *S. typhimurium* driven PMN transepithelial migration (NS, not significantly different from PMN treated with t-BOC MLP). Data are expressed as the mean and SD of triplicate samples. This experiment is representative of more than three performed.

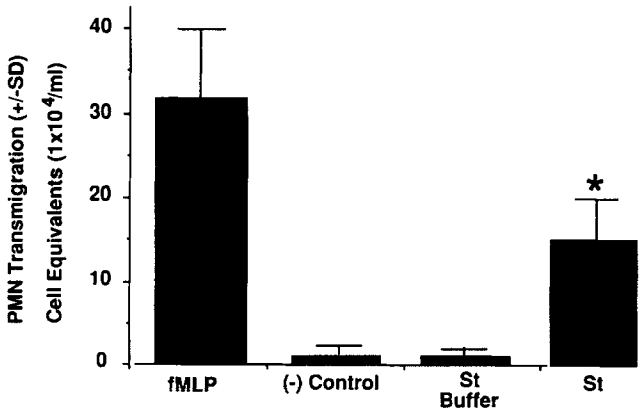

Figure 7. The effect of *S. typhimurium*-intestinal epithelial cell contact on the ability of bacteria to induce PMN transepithelial migration. *Salmonella*-induced PMN transmigration (*St*) (*$P < 0.01$) is compared to conditions in which epithelia were exposed to *Salmonella* soluble products (*St buffer*), but in which no direct *Salmonella*-epithelial cell apical membrane contact existed. *Salmonella* driven PMN transepithelial migration occurs only under conditions in which bacteria contact the apical membrane of epithelial cells. Results are expressed as mean and SD of triplicate values for each condition and are representative of one out of three experiments.

Effects of Salmonella-Epithelial Contact on Elicited PMN Transepithelial Migration

We next determined whether epithelial contact, or simply apical exposure to soluble *Salmonella* products, was essential for induction of PMN transepithelial migration. Exposure of monolayer apical membranes to *S. typhimurium* soluble products under conditions which prevented direct *Salmonella*-epithelial contact was ineffective in inducing PMN transepithelial migration (in the absence of direct bacterial-apical membrane contact PMN transmigration was reduced >fivehold [Fig. 7]).

To examine whether PMN transepithelial migration directed by cell-associated *S. typhimurium* required either apically attached or only internalized bacteria, we treated colonized monolayers with gentamicin (50 μg/ml; detailed in Materials and Methods) before beginning the PMN transmigration assay. As shown in Fig. 8, gentamicin treatment of colonized monolayers nearly ablated the subsequent PMN transepithelial migration. Gentamicin treatment alone could not explain this 87% drop in *Salmonella* induced transmigration since similar treatment of noncolonized monolayers had no effect on the ability of PMN to transmigrate in response to a fMLP gradient (1 μM) (22.31 ± 2.30 and 19.94 ± 4.60 × 10^4 PMN cell equivalents/ml, for gentamicin [50 μg/ml] treated and untreated monolayers, respectively).

Effect of Bacterial and Epithelial Protein Synthesis on the Ability of S. typhimurium to Induce PMN Transepithelial Migration

When cell attached *S. typhimurium* were exposed to chloramphenicol (Fig. 9), a bacteriostatic bacterial-specific protein synthesis inhibitor, the PMN transepithelial migration response elicited by apical colonization was 90% inhibited. Quantitation of colonized epithelial lysates revealed that such chloramphenicol exposure did not substantially affect the ability of *S. typhimurium* to adhere to T84 monolayers

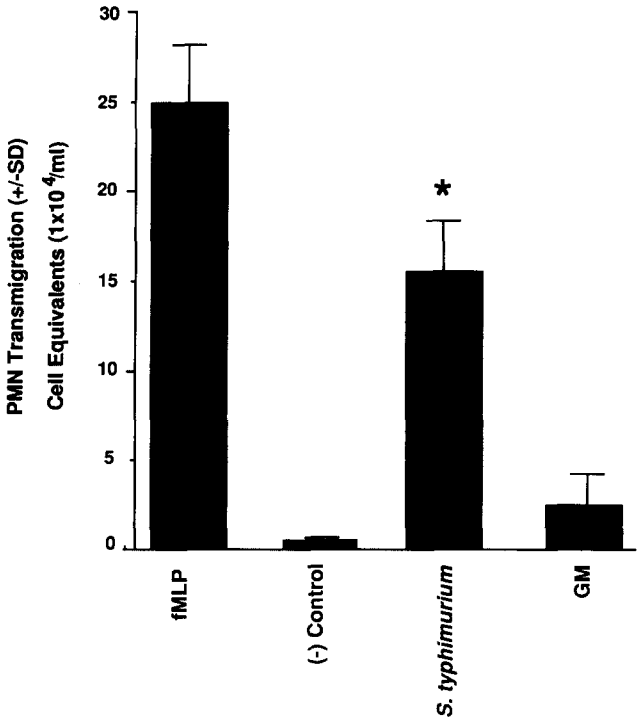

Figure 8. The effect of gentamicin on the ability of *S. typhimurium* to induce PMN transepithelial migration. *S. typhimurium* apically colonized T84 epithelial monolayers were treated with gentamicin (*GM*) (50 μg/ml) for 20 min before the addition of PMN to the basolateral surface to determine the ability of attached bacteria to drive PMN transepithelial migration. Results are obtained from harvesting the PMN specific enzyme myeloperoxidase from the lower (apical) reservoirs relative to a known standard number of PMN (*$P < 0.005$ for *S. typhimurium* minus and plus the addition of gentamicin). Results are expressed as mean and SD of three individual monolayers for each condition. Data represent one out of three experiments performed.

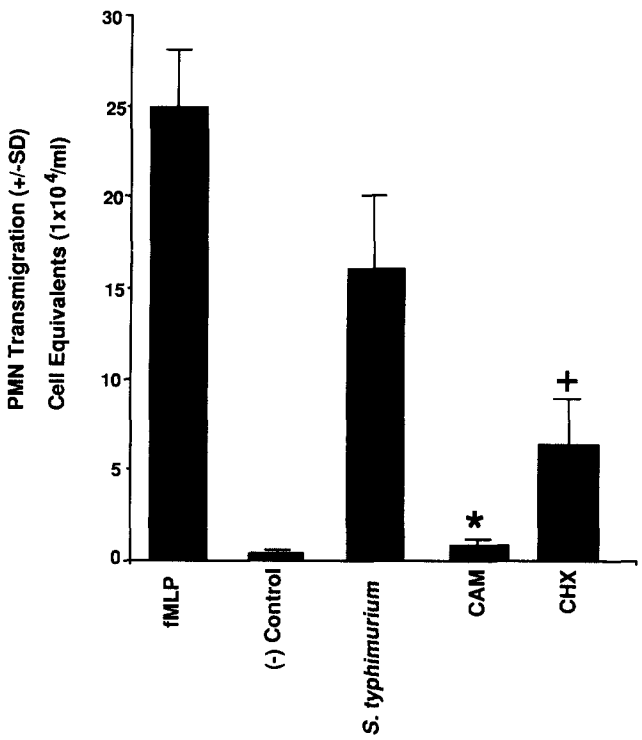

Figure 9. The effect of both *S. typhimurium*, and T84 epithelial cell protein synthesis on the ability of *S. typhimurium* to drive PMN transepithelial migration. *S. typhimurium* was treated in the presence and the absence of chloramphenicol (*CAM*), as detailed in Materials and Methods, and assessed for the ability to induce PMN transepithelial migration. As shown, CAM (100 μg/ml) treatment nearly completely inhibited the ability of *S. typhimurium* to elicit PMN transmigration (*$P < 0.005$). In a parallel study, T84 intestinal monolayers were incubated in the presence and absence of 2 μg/ml cycloheximide (*CHX*) before the addition of *S. typhimurium*. As shown, CHX treatment inhibited the ability of *S. typhimurium* to induce PMN transepithelial migration by ~50–60% (+$P < 0.05$). Results are obtained from harvesting the PMN specific enzyme myeloperoxidase from the lower (apical) reservoirs relative to a known standard number of PMN. Results are expressed as mean and SD of three individual monolayers for each condition. Data represent one out of three experiments performed.

(13.10 ± 2.46% vs 9.00 ± 0.40% for untreated versus chloramphenicol treated *S. typhimurium*, respectively). Likewise, chloramphenicol exposure (100 μg/ml) measured over 3 h, did not substantially affect the T84 transepithelial resistance (1184 ± 250 vs 1531 ± 300 ohm×cm² for chloramphenicol treated vs untreated monolayers, respectively, NS). Similarly, exposure of uncolonized monolayers to chloramphenicol had no significant influence on the ability of PMN to transmigrate in response to an imposed 1 μM transepithelial gradient of fMLP (27.40 ± 3.60 and 21.20 ± 2.17 × 10^4 PMN cell equivalents/ml for chloramphenicol-treated and -untreated monolayers, respectively). The above data strongly suggest that bacterial-epithelial apical membrane contacts and subsequent bacterial protein synthesis once contacts are established are crucial to subsequent induction of PMN transepithelial migration.

To determine whether *S. typhimurium*-elicited PMN transmigration also required epithelial protein synthesis in response to apical contact with metabolically unrestricted *Salmonella*, transmigration was assessed in the presence and absence of the eukaryotic protein synthesis inhibitor cycloheximide (2 μg/ml) (Fig. 9). Epithelial exposure to cycloheximide inhibited *S. typhimurium*-driven PMN transmigration by ~60% but by itself had no effect on the ability of PMN to transmigrate to imposed gradients of fMLP (28.20 ± 3.50 and 31.30 ± 4.2 for cycloheximide-treated and untreated monolayers, respectively, NS). It should be noted that cycloheximide did not affect the ability of *Salmonella* to adhere to these treated monolayers (11.0 ± 2.3% vs 9.5 ± 2.6% for treated vs untreated monolayers, respectively, NS), nor did this treatment significantly alter transepithelial resistances when measured after 5 h, a time point reflecting completion of the experiment (1940 ± 60 vs 2059 ± 122 ohm×cm2 for cycloheximide-treated vs -untreated monolayers, respectively). Thus, after apical colonization, cooperative metabolic "cross-talk" between bacteria and epithelial cells is required to initiate PMN transmigration.

Apical Colonization Induces Polarized IL-8 Secretion from Intestinal Epithelial Monolayers

Preliminary reports (Schurer-Maly et al., 1992) have suggested that intestinal epithelial cells may secrete the PMN

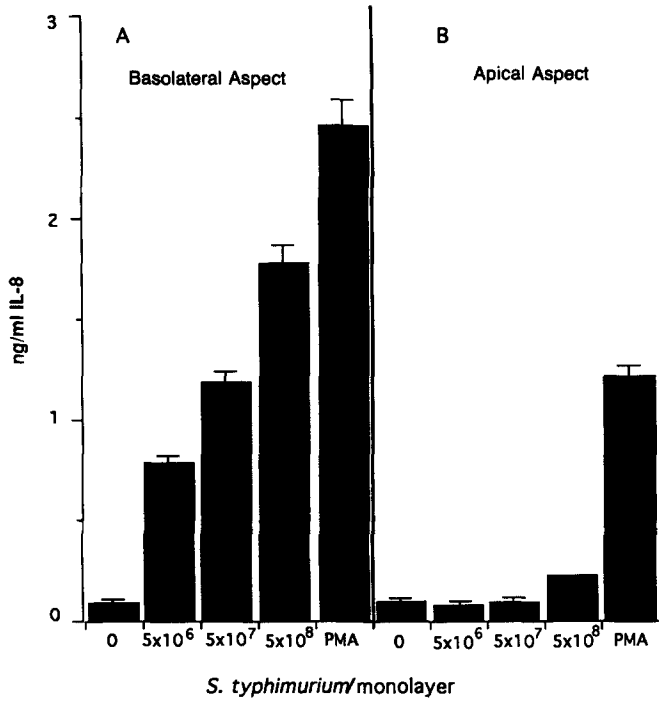

Figure 10. Effect of *S. typhimurium* on the ability to induce IL-8 secretion from T84 polarized intestinal epithelial monolayers. T84 intestinal monolayers were colonized with 0, 3, 15, and 80 cell-associated *S. typhimurium*/epithelial cell and subsequently assessed for secretion of IL-8 via ELISA assay (see Materials and Methods). Positive control monolayers were exposed to PMA (1 μg/ml). Shown here is the quantity of IL-8 secreted (ng/ml) by T84 monolayers in response to *S. typhimurium* and PMA stimulation from both the (*A*) basolateral and (*B*) apical aspect of T84 polarized monolayers. Data are expressed as the mean and SD of triplicate samples. One of three experiments performed.

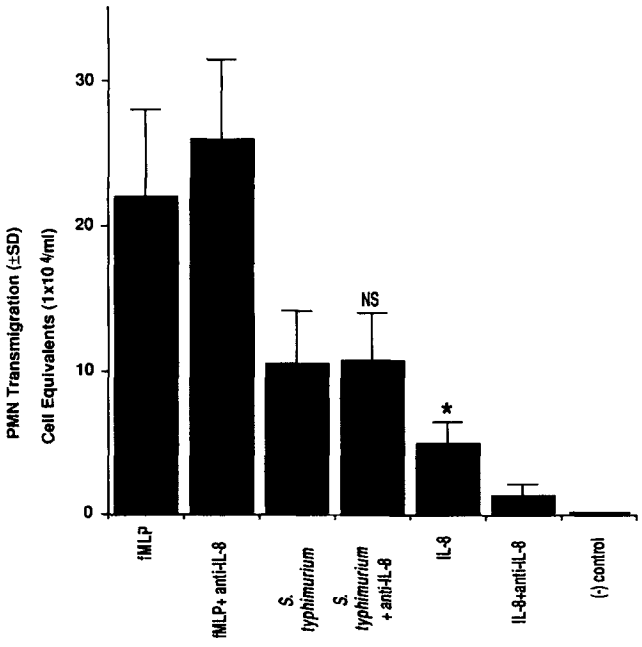

Figure 11. The effect of anti IL-8 on the ability of *S. typhimurium* to induce PMN transepithelial migration. T84 intestinal epithelial monolayers were colonized with *S. typhimurium* at a density of 80 cell associated bacteria/epithelial cell. Before the addition of 10^6 PMN/monolayer, anti-IL-8 antibody (30 μg/ml) was added to both the basolateral and apical aspect of T84 monolayers. Shown here are the results obtained from harvesting the PMN specific enzyme myeloperoxidase from the lower (apical) reservoir after 120 min to a known standard number of PMN. Positive transmigration controls are shown as PMN transepithelial migration to imposed gradients of fMLP (10^{-7} M) and IL-8 (100 ng/ml) in the presence and absence of anti-IL-8 antibody (*$P < 0.025$). Data are expressed as mean and SD of triplicate samples for each condition tested and represents one out of at least three experiments performed. NS, not significantly different from *S. typhimurium*-induced PMN transmigration in the presence of 30 μg/ml anti-IL-8 antibody.

chemoattractant IL-8 in response to signals such as phorbol esters and tumor necrosis factor. Hence, we sought to determine whether *S. typhimurium* interactions with T84 cells induced the release of IL-8. We first quantitated the release of IL-8 in response to colonization of epithelial monolayers with 3, 15, and 80 cell-associated *S. typhimurium*/epithelial cell, using the phorbol ester PMA (1 μg/ml) as a positive control (Schurer-Maly et al., 1992). As shown in Fig. 10 *a*, *S. typhimurium* colonization of monolayers induced the release of IL-8 from T84 cells in a dose-dependent manner. Furthermore, IL-8 was preferentially secreted basolaterally from these epithelial monolayers (Fig. 10 *b*), indicating that transepithelial gradients (basolateral > apical) of this cytokine could be established.

Polarized IL-8 Secretion Is Not Sufficient to Direct Neutrophil Transmigration in Response to Apical Colonization

We next determined whether the IL-8 secreted from *S. typhimurium*-stimulated T84 cells was responsible for the observed induction of PMN transepithelial migration. As shown in Fig. 11, neutralizing antibodies to IL-8 (30 μg/ml) do not inhibit transepithelial migration of PMN in response to n-formyl peptide gradients, but effectively inhibited PMN transepithelial migration induced by even large (100 ng ml^{-1}) transepithelial gradients of rIL-8. However, such neutralizing antibodies do not effectively inhibit transepithelial migration induced by apical colonization with *S. typhimurium*. Moreover, analysis of imposed rIL-8 gradients revealed that substantial gradients were necessary to elicit large-scale transepithelial migration of PMN (0.286 ± 0.265, 5.97 ± 3.50, and 28.45 ± 3.53 × 10^4 PMN cell equivalents/ml for rIL-8 imposed gradients of 5, 50, and 500 ng/ml, respectively). In contrast (Fig. 10), rIL-8 secretion induced by apical colonization by *Salmonella* yields small (<1 ng/ml) transepithelial gradients of rIL-8 which are generated opposite to the direction of PMN migration. The neutralizing activity of the rIL-8 antibody used was sufficient to completely inhibit the effect of 1 ng/ml rIL-8 gradients and neutralized 50% of the effect of 10 ng/ml rIL-8. Additionally, exogenously applied rIL-8 (ranging from 2–100 ng/ml) to the basolateral reservoir (features which mimic the IL-8 secretion pattern induced by apical surface colonization by *S. typhimurium*), does not by itself induce a substantial PMN transepithelial migration (0.319 ± 0.132 vs 0.331 ± 0.271, 0.519 ± 0.042, and 0.489 ± 0.024 PMN transmigration in cell equivalents/ml for 0 vs 2, 20, and 100-ng/ml rIL-8 ap-

plied to the basolateral surface; data not shown). In aggregate, these data indicate that while such pathogen induced IL-8 secretion likely modulates epithelial-neutrophil interactions in some fashion (see Discussion), it is not the underlying signal which induces transmigration of subepithelially positioned PMN in response to apically attached *S. typhimurium*.

Discussion

While the events related to PMN trans*end*othelial migration are widely studied (Arnaout, 1990; Dana et al., 1984; Luscinskas et al., 1989; Smith et al., 1988), those related to trans*epi*thelial migration have received less attention (Cramer et al., 1986; Evans et al., 1983; Migliorsi et al., 1988; Parsons et al., 1987). In the intestine, transepithelial migration is of particular importance since patient symptomatology often correlates with the degree of neutrophil transepithelial migration (Teahon et al., 1991). Moreover, recent data suggest that subsequent to transepithelial migration, neutrophils may influence intestinal epithelial functions by interacting with targets polarized to the apical membrane domain (Madara et al., 1993). This study defines an in vitro model system of *S. typhimurium* interactions with human intestinal epithelial cell monolayers. Using this model, we demonstrate that surface attached *Salmonella* can trigger subsequent physiologically directed PMN emigration.

Salmonella Interactions with Intestinal Epithelia

Salmonella may interact with the apical membranes of columnar intestinal epithelia (Takeuchi, 1967) or with specialized cells, termed M cells, which lie over the Peyer's patches (Kohbata et al., 1986; Pospischil et al., 1990; Takeuchi, 1967). The relative contributions of these interactions to the pathogenesis of salmonellosis is uncertain. In mice, evidence suggests that early entry of *S. typhimurium* appears to be via transepithelial transport by M cells to the Peyer's patches (Hohmann et al., 1978; Kohbata et al., 1986). Subsequent to M cell entry, enterocytes are invaded by *S. typhimurium*, providing an additional portal of entry (Takeuchi, 1967). M cells are a minor constituent of the epithelial surface area (far less than 1%) (Trier and Madara, 1988), however they appear to be the entry pathway initiating the afferent limb of the intestinal immune response (Kagnoff, 1988). Additionally, some pathogens which are unable to associate with or translocate across columnar intestinal epithelia use the M cell pathway to enter the host (for instance, reovirus) (Wolf et al., 1983). Our model does not account for such *Salmonella*-M cell interactions. Columnar epithelial cells of the intestine constitute the major portion of surface area ▯ (Trier and Madara, 1988), are known to bind *Salmonella* and internalize it, and are the site at which PMN transmigration in response to such surface colonization is known to occur (Day et al., 1978; McGovern and Slavutin, 1979; Rout et al., 1974; Takeuchi, 1967). Hence, although M cell-*Salmonella* associations may play a role in host immunity to this organism, the bulk of prokaryotic-eukaryotic interactions in primary colonization of the intestine by *Salmonella* likely occurs over the general columnar epithelial surface as suggested by the structural studies of Takeuchi (1967). *Salmonella* binding to epithelial cells engages a complex signaling cascade which includes cytoskeletal reorganization, changes in $[Ca^{++}]i$, and perhaps activation of kinases as a result of binding to specific apical receptors (Galan et al., 1992*b*). Although such information is derived from renal cell lines, the cytoskeletal reorganization reported in these renal models mimics both that originally found in studies of intestinal salmonellosis and in the current studies which use natural target of an intestinal epithelium.

Salmonella-induced Directed PMN Migration and the n-formyl-peptide Receptor

N-formylated peptides are metabolic products of bacteria which are not formed by eukaryotic cells. Receptors for such peptides on the surface of PMN thus allow for directed migration of inflammatory cells to sites of infection (Snyderman and Uhing, 1988). Indeed, although many bacterial products activate neutrophils, n-formyl-peptides are the major known bacterial product which induce directed migration (Gallin, 1988). It is not surprising then, that it is often implicitly suggested that such n-formyl-peptide mediated events underlie PMN transepithelial migration associated with pathogens such as *Salmonella*. For example, the pathology of non-typhoidal *Salmonella* infections in man reveals massive PMN infiltration into both the large and the small bowel (Gallin, 1988; Rout et al., 1974). A confounding twist to the role played by n-formyl-peptides in such states was added by the recognition that the human intestinal lumen appears to naturally contain micromolar concentrations of n-formyl-peptide due to the heavy bacterial load which is uniquely present at this site (Chadwick et al., 1988). Recognition of this fact would seem to mitigate against the use of this classical pathway to organize pathogen-driven directed PMN migration at this site in the presence of an intact epithelial barrier. On the other hand, if pathogens such as *Salmonella* were able to modulate epithelial permeability as a result of surface attachment, perhaps the natural transepithelial gradient could then suffice to drive transepithelial migration down the newly apparent, but endogenous, n-formyl-peptide gradient. Indeed, in Crohn's disease, a disorder which has no known lumenal pathogen but is characterized in its active phase by transepithelial migration of PMN into the intestinal lumen, it has recently been speculated that genetically determined abnormalities in barrier function may permit neutrophil transmigration via the endogenous n-formyl peptide gradient (Hollander, 1989). The importance of such unmasking of the naturally occurring transepithelial n-formyl peptide gradient is potentially relevant to salmonellosis since it has been recognized that epithelial barrier function is modestly influenced several hours after heavy colonization of the apical surface (Finlay et al., 1988). Here however, we show that, under conditions in which the epithelial barrier remains intact, apically bound *Salmonella* can nevertheless signal to subepithelial PMN and drive PMN efflux. Moreover, we use a n-formyl-peptide receptor antagonist to demonstrate that apically attached *Salmonella* may drive directed neutrophil migration by means other than that involving the n-formyl-peptide receptor.

Salmonella Attachment to the Apical Membrane of T84 Intestinal Epithelial Monolayers Induces Polarized Secretion of the PMN Chemoattractant and Activator IL-8

Evidence is emerging that bacterial binding to eukaryotic

cells can influence the program of transcriptional regulation for synthesis of biologically important eukaryotic products. For example, IL-6 production is stimulated by the binding of adherent *E. coli* to bladder or kidney epithelial cells (Hedges et al., 1991), and LPS has been shown to stimulate tumor necrosis factor (TNF), IL-1, IL-6, and IL-8 production in a host of cell types including monocytes, fibroblasts, and endothelial cells (Jirik et al., 1989; Lippnow et al., 1990; Takada et al., 1991). IL-8 is of particular potential significance for, unlike the other cytokines listed, it is a potent PMN chemotaxin when present as a gradient (Huber et al., 1991). A recent report (Agace et al., 1993) modeling urinary tract infections (UTIs) also provided direct evidence that urinary epithelial cells exposed to *E. coli* secrete IL-8 and such observations fit well with studies indicating that IL-8 can be recovered from the urine of patients suffering from UTIs (Ko et al., 1993). Although similar studies are not available from intestinal epithelia, a preliminary report by Schurer-Maly and coworkers (1992) showing that intestinal epithelia could secrete IL-8 in response to PMA or TNF-α indicated that this epithelium is also capable of regulated IL-8 secretion.

We demonstrate that *S. typhimurium*-T84 intestinal epithelial interactions not only induce the release of IL-8 in a dose-dependent fashion but also promote IL-8 secretion in a polarized, preferentially basolateral, manner. While such cytokine secretion is likely involved in intestinal inflammation (see below), the improperly directed gradient does not explain basolateral-to-apically directed PMN transepithelial migration. This interpretation was substantiated by additional experiments which indicated that *Salmonella*-elicited transepithelial migration is not ablated by neutralizing antibodies to IL-8, is not induced by mimicking IL-8 concentrations with exogenously added IL-8, and is not mimicked by conditions which elicit comparable IL-8 synthesis from epithelial cells.

Reciprocal Metabolic Interactions Are Required for Salmonella Signaling to PMN Across Epithelial Monolayers

The above findings indicate that apically bound *Salmonella* can signal across intact intestinal epithelial monolayers to underlying neutrophils and appear to do so via a novel pathway which does not involve classic bacterial-derived chemotaxins such as n-formyl-peptides or the chemotactic cytokine IL-8. Furthermore, not only is attachment to the apical membrane required to initiate this response, but bacterial protein synthesis is necessary as well. This observation fits well with the recent appreciation of the regulation of some of the virulence factors necessary for *Salmonella* pathogenesis (Alpuche Aranda et al., 1992; Lee and Falkow, 1990; McBeth and Lee, 1993). For example, recent studies indicate that specific bacterial genes can be transcriptionally activated within macrophages but not in epithelial cells (Alpuche Aranda et al., 1992). Further, the findings of Lee and Falkow (1990) suggest that anaerobiosis is a signal for the synthesis of labile factors necessary for *S. typhimurium* invasion (McBeth and Lee, 1993). Assuming such is the case for *Salmonella* attachment to intestinal epithelia, it is likely that recognition of these newly expressed gene products leads to reciprocal alterations in protein expression by the epithelial cells. Such reciprocal metabolic interactions are suggested by the finding that *Salmonella*-driven PMN transepithelial migration is also suppressed if epithelial protein synthesis is inhibited after apical attachment to intestinal epithelial cells.

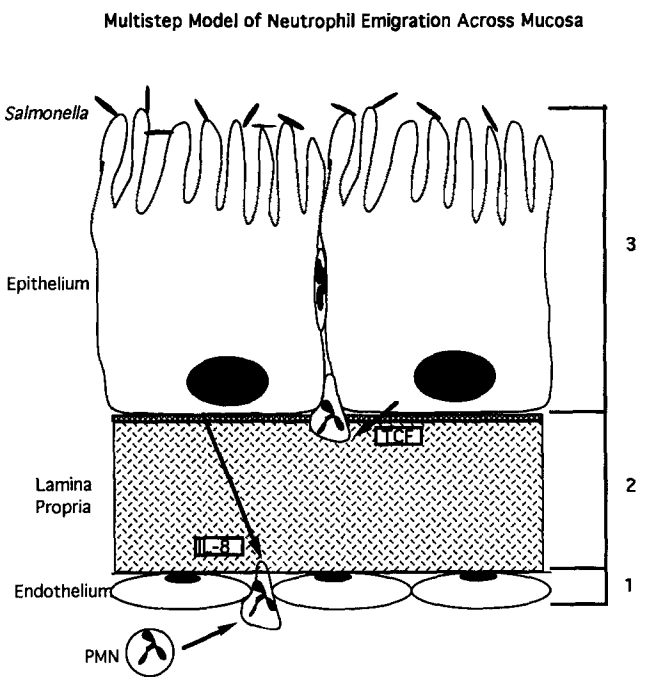

Figure 12. Multistep model of neutrophil emigration across intestinal mucosa in response to apically attached *Salmonella*. Emigration of PMN can be conceptually divided into three events: (*1*) microvascular emigration, (2) transmigration across the lamina propria matrix and, (*3*) transepithelial migration (see Discussion). Known distorting effects of solvent flow in the microenvironment of the lamina propria would seem to favor long range matrix binding chemotactic signals for the second event. Speculatively, basolateral epithelial release of IL-8 in response to apically attached *Salmonella* might serve this role well (see text). However, such IL-8 secretion is not sufficient to stimulate transepithelial migration. Thus, for the final migratory event, novel transcellular chemotactic factor (*TCF*) activities generated in response to apically attached *Salmonella* are required.

A Multi-step Model of PMN Emigration Across Intestinal Mucosa

Based on previous observations as well as the current data, we present a working model of PMN efflux from the mucosa such as is seen in response to epithelial colonization by *S. typhimurium* (Fig. 12). Such PMN efflux might, for convenience, be separated into three major events: (*a*) the well recognized rolling, firm adhesion, and subsequent emigration of neutrophils from the microvasculature (Arnaout, 1990; Butcher, 1991; Dana et al., 1984; Luscinskas et al., 1989; Smith et al., 1988); (*b*) subsequent migration of PMN across the lamina propria and into a subepithelial position; and (*c*) transepithelial migration. One unique aspect of the intestinal mucosa which might require different signals for the latter two events is the presence of a vascular countercurrent arrangement in the subepithelial compartment (Jodal and Lundgren, 1986). As happens for absorbed solutes (Jodal and Lundgren, 1986), this countercurrent phenomenon may distort transepithelial solute gradients. For example, we have found that perfusion of mammalian intestinal loops in vivo

with solutions containing fMLP induces neutrophil attachment to endothelial cells and structurally defined endothelial activation, but fails to elicit directed migration across the lamina propria (Nash, S., J. L. Madara, unpublished observations). Such observations suggest that directed migration across the lamina propria and into the subepithelial position may require a more stable gradient than that afforded by the usual soluble signals. We speculate that epithelial-derived signals such as IL-8 may be useful for this purpose, since this chemotaxin has the unique ability to bind avidly to matrix molecules and is also extremely resistant to degradation (Baggiolini et al., 1992). Thus gradients of IL-8 formed across the lamina propria matrix would likely be relatively resistant to the distorting effects of the complex solvent flow patterns which exist in this microenvironment and could serve to bring PMN into the subepithelial space (event 2 of Fig. 12). However, as we show, basolaterally polarized release of IL-8 by itself is not likely to be sufficient to drive the final event of transepithelial migration of PMN. Rather, other signals, such as those derived from apically attached pathogens, are likely needed for the final event of transepithelial migration. This latter transcellular chemotactic factor which we are now attempting to isolate, could act either in gradient fashion (i.e., polarized apical secretion), by promoting surface haptotaxis from the basolateral to the apical membrane, or via another means altogether. It remains to be seen if the similar transcellular recruitment of PMN by apically attached pathogens might also apply to other organisms which foster acute infectious gastroenteritis such as *Shigella sp, Yersinia sp*, or enteropathogenic *E. coli* (EPEC).

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