

# Surface Attachment of *Salmonella typhimurium* to Intestinal Epithelia Imprints the Subepithelial Matrix with Gradients Chemotactic for Neutrophils

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**Abstract.** During intestinal disease induced by *Salmonella typhimurium* transepithelial migration of neutrophils (PMN) rapidly follows attachment of the bacteria to the epithelial apical membrane. Among the events stimulated by these interactions is the release of chemotaxins that guide PMN through the subepithelial matrix and subsequently through the epithelium itself (McCormick, B. A., S. P. Colgan, C. Delp-Archer, S. I. Miller, and J. L. Madara. 1993. *J. Cell Biol.* 123:895–907). Given the substantial volume flow that normally characterizes matrix compartments underlying transporting epithelia, it is unclear how such transmatrix signaling is sustained. Here we show that when underlying matrices are isolated from biophysically confluent polarized monolayers of the human intestinal epithelial cell line T84, they fail to support substantial transmatrix migration of PMN unless an exogenous chemotactic gradient is imposed. In contrast, such matrices isolated from confluent monolayers apically colonized with *S. typhimurium* support spontaneous transmatrix migration of PMN. Such chemotactic imprinting of underlying matrices is resistant to volume wash and is paral-

leled by secretion of the known matrix-binding chemokine IL-8. Chemotactic imprinting of the matrix underlying *S. typhimurium*-colonized monolayers is dependent on epithelial protein synthesis, is directional implying the existence of a gradient, and is neutralized by antibodies either to IL-8 or to the IL-8 receptor on PMN. An avirulent *S. typhimurium* strain, PhoP<sup>c</sup>, which attaches to epithelial cells as efficiently as wild-type *S. typhimurium*, fails to induce basolateral secretion of IL-8 and likewise fails to imprint matrices. Together, these observations show that the epithelial surface can respond to the presence of a luminal pathogen and subsequently imprint the subepithelial matrix with retained IL-8 gradients sufficient to resist washout effects of the volume flow that normally traverses this compartment. Such data further support the notion that the primary role for basolateral secretion of IL-8 by the intestinal and likely other epithelia is recruitment of PMN through the matrix to the subepithelial space, rather than directing the final movement of PMN across the epithelium.

**C**OLONIZATION of mucosal surfaces by pathogenic bacteria often results in transepithelial migration of neutrophils. For example, in the intestine, transepithelial migration of neutrophils rapidly follows attachment of the *Salmonella typhimurium* to the epithelial apical membrane (Takeuchi, 1967), and this neutrophil response occurs before loss of epithelial structural integrity (Day et al., 1978). While such relationships have been observed for over three decades, the details of how *Salmonella*-intestinal epithelial contacts evoke this classic histological response

are not well-characterized (Takeuchi, 1967; Rout et al., 1974; McGovern and Slovutin, 1979). However, it is clear that mucosal surfaces have evolved the ability to mount a cellular response to pathogens even while such challenges are restricted to the lumen. How might such transepithelial signaling of underlying inflammatory responses occur? The transepithelial migration of neutrophils in response to luminal pathogens necessarily involves movement through several anatomic compartments, each with their own complexities. Initially neutrophils must emigrate from the microvasculature (Pober et al., 1986; Osborn, 1990; Springer, 1990) to the subepithelial lamina propria. This process is perhaps one of the best understood in terms of the molecular interactions that correspond to the initial tethering

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(selectin mediated) and subsequent firm attachment ( $\beta 2$  integrin mediated) of neutrophils to the endothelial surface (Dana et al., 1984; Smith et al., 1988; Luscinskas et al., 1989; Arnout, 1990; Butcher, 1991). Ultimately, neutrophils must cross the epithelial lining of the intestine to encounter the surface-attached pathogen and initiate mucosal defense. This process, too, is beginning to be understood at a more mechanistic level (McCormick et al., 1993, 1995). However, while the biology of neutrophil emigration from the vasculature is reasonably understood and that of neutrophil transepithelial migration is beginning to be understood, how neutrophils migrate across the intervening matrix remains a mystery. One of the confounding features of such matrices in organs as diverse as the kidney and intestine is the exceedingly high volume of flow through the subepithelial spaces resulting from an array of active absorptive and secretory processes. Such fluid flow exerts substantial sweeping away effects on solute gradients within subepithelial matrices. For example, in the intestine, due to the unique arrangement of its vasculature, countercurrent exchange results in rapid distortion of solute gradients in the subepithelial space (Jodal and Lundgren, 1986). Consistent with this fact, we have observed that infusion of a small water-soluble neutrophil chemoattractant in the intestinal lumen of animals induces neutrophil recruitment at the level of the microvasculature, but these recruited neutrophils fail to migrate across the matrix to the subepithelial space (Madara, J. L., unpublished observations), likely due to the distorting influences of countercurrent forces on gradients of water soluble solutes in this subepithelial compartment (Jodal and Lundgren, 1986). One potential explanation for the successful movement of neutrophils across such matrices in natural inflammatory states is that transmatrix migration might be directed by gradients that are matrix fixed.

We have recently used the human intestinal epithelial cell line T84 to model neutrophil-intestinal epithelial interactions (Dharmasathaphorn and Madara, 1990; Madara, 1990; McCormick et al., 1993) and to investigate the influence of *S. typhimurium* contact with the intestinal epithelial surface on the subsequent inflammatory response (McCormick et al., 1993, 1995). As occurs in intact tissue, we find surface attachment of *S. typhimurium* to T84 monolayer apical membranes stimulates transepithelial migration of neutrophils while the epithelium is still intact, as assessed by structural and biophysical criteria (McCormick et al., 1993). We (McCormick et al., 1993) and others (Eckmann et al., 1993) have shown that basolateral secretion of IL-8 occurs as a result of these interactions, but it is unclear how such IL-8 secretion contributes to the events of transepithelial migration. Another chemokine with novel physical characteristics is also secreted apically as a result of *S. typhimurium*-epithelial contact and appears to be sufficient for the movement of subepithelial neutrophils across the epithelium and into the luminal compartment (McCormick, B. A., and J. L. Madara, manuscript in preparation). It is unclear if such pathogen-stimulated release of chemotactic bioactivities from epithelial cells might also provide for retained gradients in the subepithelial matrix—an event that might be required to link transendo—with transepithelial migration. If such events occur, then one should be able to imprint matrices underlying epithelial cells with chemotactic memories resistive of sweep-

ing away effects of solvent flow. Here we demonstrate that apical association of pathogenic *S. typhimurium* with model intestinal epithelial monolayers results in imprinting of a neutrophil chemotactic signal on the underlying matrix. The imprinted signal resists wash out and is largely due to polarized basolateral secretion of C-X-C family member IL-8. These findings suggest the role of basolateral IL-8 secretion may be to guide transmatrix migration, and are consistent with the ability of C-X-C family members to tightly bind to matrix components such as glycosaminoglycans (Baggiolini et al., 1994).

## Materials and Methods

Approximately 750 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 (Dharmasathaphorn and Madara, 1990) with recently detailed modifications (Madara et al., 1992). T84 cells are grown as monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium supplemented with 15 mM HEPES buffer (pH 7.5), 14 mM NaHCO<sub>3</sub>, 40 mg/ml penicillin, 8 mg/ml ampicillin, 90 mg/ml streptomycin, and 5% newborn calf serum. Monolayers were grown on 0.33-cm<sup>2</sup> suspended polycarbonate filters (Costar Corp., Cambridge, MA) and used 6–14 d after plating, as described previously (Madara et al., 1992). A steady-state resistance (~1,500 ohm cm<sup>2</sup>) is reached in 4–6 d with variability largely related to cell passage number. Monolayers received one weekly feeding after initial plating. Inverted monolayers, used to study transmigration of neutrophils in the physiological matrix-to-lumen direction were constructed as described before (Nash et al., 1991; Madara et al., 1992; Parkos et al., 1992).

### Bacterial Strains and Growth Conditions

**Bacterial Strains.** *S. typhimurium*  $\chi 3306$  is a naladixic acid-resistant (*gyrA1816*) strain derived from *S. typhimurium* strain SR-11 (Gulig and Curtiss, 1987). *S. typhimurium* PhoP<sup>c</sup> (strain CS022) carries a constitutive mutation within the *S. typhimurium* PhoP regulatory locus (*pho-24*, and phenotype PhoP<sup>c</sup>) that results in repression of PhoP repressed genes (Miller and Mekalanos, 1990; Miller, 1991; Behlau and Miller, 1993).

**Media.** Luria broth was made as previously described by Revel (1966). L agar is Luria broth containing 12 g of bacto agar (Difco Laboratories, Detroit, MI) per liter.

**Bacterial growth conditions.** Nonagitated 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 (~18 h) at 37°C, as previously detailed (McCormick et al., 1993). Bacteria from such cultures were in late logarithmic phase of growth and correlated with 5–7 × 10<sup>8</sup> (colony forming units/ml) routinely.

### *S. typhimurium* Interactions with Polarized T84 Apical Surfaces

Infection of polarized T84 monolayers by *S. typhimurium* was performed by the method of McCormick et al. (1993), and is briefly described. Inverted T84 monolayers (Nash et al., 1991; Parkos et al., 1992) were rinsed extensively in HBSS(+) (containing Ca<sup>2+</sup> and Mg<sup>2+</sup>, with 10 mM HEPES, pH 7.4 (Sigma Chemical Co., St. Louis, MO), to remove residual serum components. Overnight cultures of *S. typhimurium* (grown as described above) were prepared by washing in HBSS(+) physiologic buffer, and resuspended to a final concentration of 5 × 10<sup>9</sup> bacteria/ml. Approximately 5 × 10<sup>8</sup> bacteria in 25  $\mu$ l were gently distributed onto the apical surface and incubated for 45 min at 37°C. Nonadherent bacteria were subsequently removed from the monolayers by washing and were then transferred back into the 24-well tissue culture tray containing 1.0 ml HBSS(+) buffer in each lower reservoir (apical membrane now colonized with *S. typhimurium*), and 140  $\mu$ l in the upper reservoir (basolateral interface).

## Preparation of *S. typhimurium*-Epithelial-conditioned Matrix on Polycarbonate Filters

T84 cells were plated onto polycarbonate filters which were coated with rat tail collagen (50  $\mu$ l of 1:100 dilution of viscous rat tail collagen in ethanol) and allowed to dry before plating. Although low concentrations of rat tail collagen are required for efficient attachment to filters, in the 5–7 d until physiological confluence is achieved these cells lay down a matrix  $\sim$ 1–2  $\mu$ m in thickness on which electrically confluent polarized monolayers reside (Madara et al., 1987). *S. typhimurium*-T84 epithelial cell-conditioned matrix was prepared by a slight modification of the method previously described by Schubert and LaCorbiere (1980). As illustrated in Fig. 1, T84 cell monolayers apically colonized with *S. typhimurium* (prepared as above), were transferred to fresh 24-well plates containing a solution of  $5 \times 10^{-4}$  M ethylene glycol-bis( $\beta$  aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA; Sigma Chemical Co.) in HBSS(-) (without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , with 10 mM HEPES, pH 7.4 (Sigma Chemical Co.), at 37°C. T84 cell monolayers were next gently stripped from the collagen-coated polycarbonate filters via bathing in EGTA-HBSS(-) for 2 h at 37°C with gentle agitation. After the first hour of EGTA treatment, the T84 cells were washed twice with  $\sim$ 6–10 ml of HBSS(-), and returned to fresh wells containing EGTA-HBSS(-) where they were incubated for the remaining (second) hour. The monolayers were then viewed under an inverted phase microscope (IM35; Carl Zeiss, Inc., Thornwood, NY) to verify the filters were void of epithelial cells. In addition, confocal microscopy using rhodamine-conjugated phalloidin to detect epithelial filamentous actin confirmed the uniform removal of monolayers using this approach. Lastly, the inverts were washed with  $3 \times 150$ -ml washes in HBSS(+) and transferred back into the original 24-well tissue culture trays containing 1.0 ml HBSS(+) buffer in the lower reservoir (apical interface), and 100  $\mu$ l in the upper reservoir (basolateral interface). Control matrices consisted of those laid down by T84 cell monolayers that had not been colonized with *S. typhimurium*. Thus, matrices finally isolated had received several high volume washes, sufficient to remove soluble signals such as  $^3\text{H}$  mannitol (10  $\mu\text{Ci}/\text{ml}$ ) from filter matrices. Specifically,  $3 \times 150$ -ml washes reduced counts of radioactivity to background levels, while unwashed control filter matrices were determined to be 250 times background.

## Neutrophil Transepithelial Migration across Conditioned Matrix

Neutrophils obtained from normal human volunteers were isolated from anticoagulated (sodium citrate, 13.2 g, and dextrose, 11.2 g in 500 ml water, pH 6.5) whole blood using the gelatin sedimentation technique, as previously described (Henson and Oades, 1975; Parkos et al., 1992).  $10^6$  isolated neutrophils suspended in a 20- $\mu$ l vol were added to the basolateral bath (upper reservoir since monolayers were grown in an inverted fashion). Incubations were for 90 min at 37°C unless specifically indicated otherwise. Positive control transmigration assays were performed by imposing chemoattractant gradients ( $10^{-7}$  M *n*-formyl methionylleucylphenylalanine)<sup>1</sup>. At the end of each experiment, monolayers were cooled to 4°C, and neutrophil transmigration was quantitated by assaying for the neutrophil-specific azurophilic granule marker myeloperoxidase as described previously (Parkos et al., 1991). Neutrophil cell equivalents (CE), estimated from a standard curve, were assessed as the number of neutrophils that had completely traversed the matrix/polycarbonate filter (i.e., into the lower reservoir). Numbers of neutrophils associated with the matrix are not presented since they are a minor fraction of the transmigrated population. The standard curve was linear in the range used (0.30–50  $\times 10^4$  cells/ml).

## Chloramphenicol Treatment of *S. typhimurium* and Cycloheximide Treatment of T84 Monolayers

In the indicated subsets of experiments, chloramphenicol (100  $\mu\text{g}/\text{ml}$ ), a bacteriostatic protein synthesis inhibitor (which does not similarly affect eucaryotic cells) was added to *S. typhimurium* (McBeth and Lee, 1993) before the 45-min colonization step, and remained in both the apical and basolateral reservoirs throughout the course of neutrophil transmigration (McCormick et al., 1993). Where used, cycloheximide (Sigma Chemical Co.), an inhibitor of eucaryotic but not prokaryotic protein synthesis was

1. Abbreviations used in this paper: CE, cell equivalent; fMLP, (*n*-formyl) formylmethionylleucylphenylalanine.

prepared as a 2-mg/ml stock in 95% ethanol and diluted to a final concentration of 2  $\mu\text{g}/\text{ml}$  in tissue culture media. This concentration inhibits 75% of radiolabeled leucine incorporation into T84 cell-precipitable protein (McRoberts et al., 1990).

## 4°C Treatment of T84 Cell Monolayers

*S. typhimurium* were washed in HBSS(+) buffer as described above and equilibrated to 4°C. Inverted monolayers were washed in HBSS(+) at 37°C, as indicated above, and like *S. typhimurium*, were equilibrated to 4°C. The cold bacterial suspension was then gently distributed onto the cold apical surface of the T84 cell monolayer, and incubated at 4°C for 45 min. The nonadherent bacteria were removed by washing thrice in HBSS(+), and the inverted monolayers were transferred into a fresh 24-well tray containing EGTA-HBSS(-), as described above, at 4°C. T84 cell monolayers were effectively removed from the collagen-coated polycarbonate filters at either 37 or 4°C. After the 2-h treatment, the inverts were then washed in HBSS(+) and transferred back into 24-well tissue culture trays containing HBSS(+), and the plates transferred to 37°C. After the conditioned filters warmed to 37°C, neutrophil transmigration across the conditioned matrix was assessed as previously detailed above.

## Anti-IL-8 Antibody Studies

For neutralizing polyclonal antibodies against human IL-8 (Endogen, Inc., Boston, MA) 30  $\mu\text{g}/\text{ml}$  were exposed to the filter matrix after EGTA

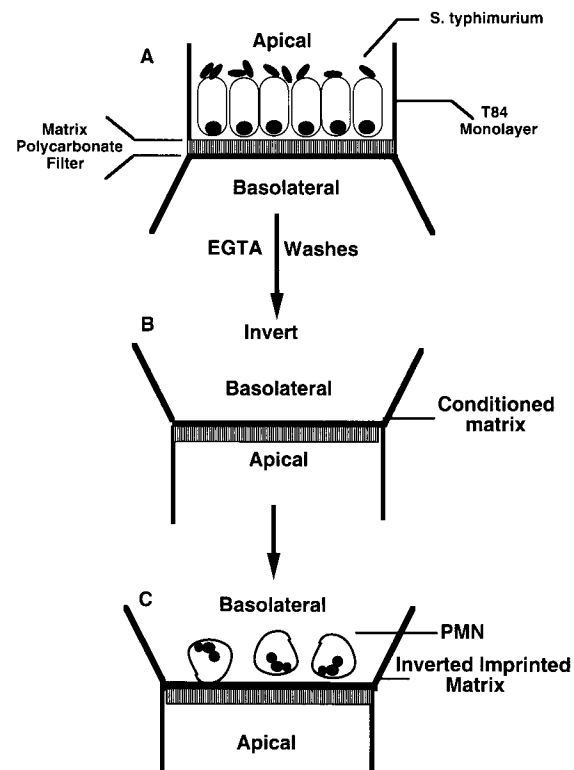


Figure 1. Preparation of *S. typhimurium*-epithelial-conditioned matrix on polycarbonate filters. (A) T84 monolayers, which lay down their own complex matrix (Madara et al., 1987) after being plated on rat tail collagen-coated permeable supports, were grown to confluency and subsequently the apical membranes were colonized by *S. typhimurium*. This procedure was done under conditions in which the monolayer maintains biophysical polarity and thus basolaterally secreted proteins are transferred to the matrix. (B) Monolayers were then stripped and the isolated matrices washed. (C) The stripped imprinted matrices were inverted so that basolateral-to-apical-directed transmigration could be assessed. Details of this protocol are described in Materials and Methods.

treatment, as described previously (McCormick, 1993), for 30 min before the addition of  $10^6$  neutrophils/well. This concentration of antibody was chosen since secretion of IL-8 induced by apical colonization by *S. typhimurium* yields small ( $<1$  ng/ml) transepithelial gradients of IL-8 that are generated opposite to the direction of neutrophil migration (McCormick et al., 1993). The neutralizing activity of the rIL-8 antibody used was sufficient to inhibit completely the effect of 1 ng/ml rIL-8 gradients and neutralized 50% of the effect of 10 ng/ml rIL-8 (McCormick et al., 1993). Neutralizing mAbs against the IL-8 receptor (IL-8 receptor A) on neutrophils were used via a slight modification by Chuntharapai et al. (1994). Specifically, 50  $\mu$ g/ml antibody was added to  $5 \times 10^7$  neutrophils, prepared as described above, but containing 0.5% BSA in HBSS(-). The addition of BSA to the neutrophils had no effect on the transmigrating ability of the neutrophils. The neutrophil-mAb mixture was then gently rotated on a shaker at 4°C for 90 min to prevent settling of the neutrophils.  $10^6$  neutrophils were added to upper reservoir of conditioned matrix/polycarbonate filters. After a 90-min incubation period at 37°C, neutrophils were assessed for their ability to migrate across the underlying matrix via an enzymatic assay for the specific marker myeloperoxidase (Parkos et al., 1991, 1992).

### Conditioning Control Matrices with rIL-8

Control matrices, prepared as described above, consisted of those laid down by T84 monolayers that had not been colonized with *S. typhimurium*. To condition a subset of these matrices with exogenous IL-8, these matrices were exposed to gradients of either 0, 2, 20, or 200  $\mu$ g/ml of human rIL-8 (R and D Systems, Minneapolis, MN) applied apically for ei-

ther 45 or 90 min at 37°C (apical > basolateral). After each time point, the conditioned matrices were washed three times in 150-ml vol of HBSS(+) buffer for both the top and bottom reservoirs. Transmatrix migration of neutrophils was subsequently assessed in both the presence and absence of neutralizing polyclonal anti-IL-8 antibodies, as described above.

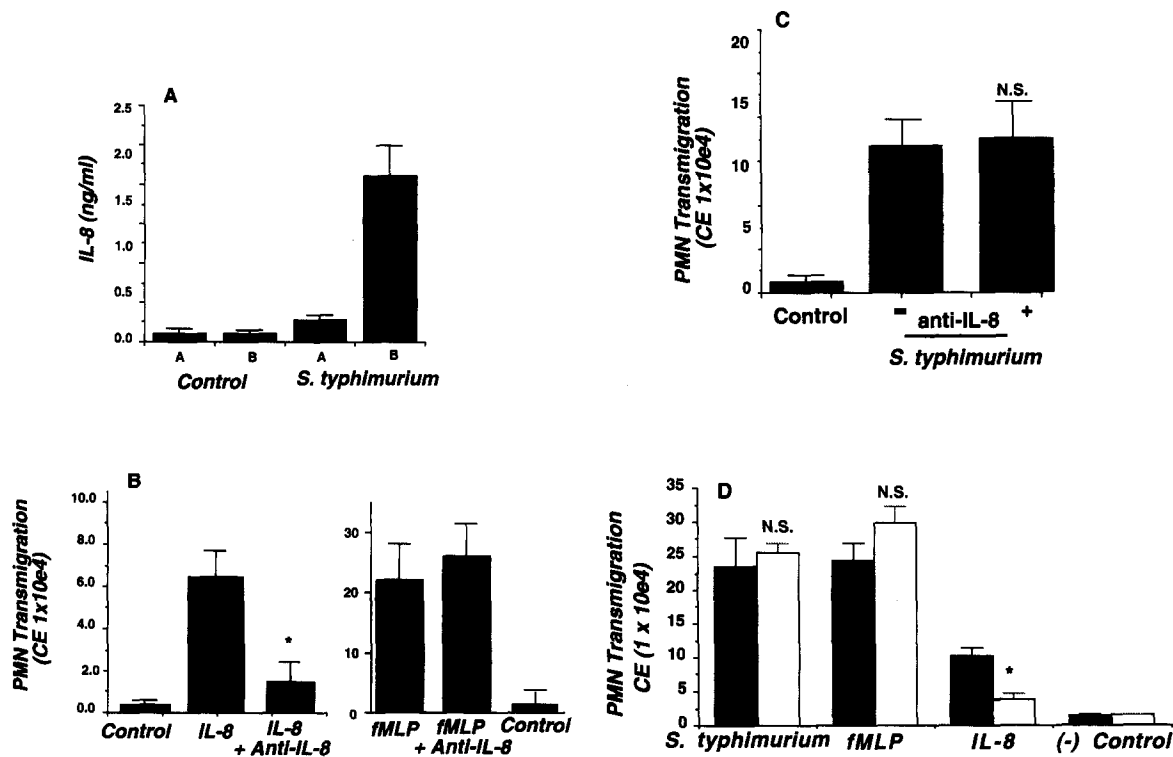
### Data Presentation

Neutrophil isolation was restricted to 10 different donors (repetitive donations) over the course of these studies. Myeloperoxidase assay (i.e., neutrophil transmigration) data were compared by Student's *t* test. Neutrophil transmigration results are represented as neutrophil CE derived from a daily standard neutrophil dilution curve. Neutrophils that completely traversed the filter are represented as the number of neutrophils CE/ml (total vol 1 ml). Values are expressed as the mean and SD of an individual experiment done in triplicate repeated *n* times.

### Results

#### Basolaterally Secreted IL-8 in Response to Apical Salmonella Attachment Does Not Drive the Final (i.e., Postmatrix) Event of Transepithelial Migration of Neutrophils

As previously shown (Eckmann et al., 1993; McCormick et al., 1993) apical membrane colonization with *S. typhimu-*



**Figure 2.** The effects of *S. typhimurium* interactions with polarized T84 monolayers on transepithelial migration. In the subset of experiments described, the T84 epithelial cell monolayers remained intact on the filter matrix. (A) T84 intestinal monolayers were colonized with 50 cell-associated *S. typhimurium*/epithelial cells and assessed for polarized secretion of IL-8 in control T84 monolayers or monolayers colonized by *S. typhimurium* (50 bacteria/cell). IL-8 secretion was assayed by ELISA as previously described (McCormick et al., 1993). A, secretion into the apical reservoir; B, secretion into the basolateral reservoir. (B) Anti-IL-8 antibodies (30  $\mu$ g/ml) effectively ablate transepithelial migration of neutrophils across T84 monolayers in response to imposed maximal gradients of rIL-8 (100 ng/ml) but not fMLP ( $10^{-7}$  M) (\* $P < 0.025$  for IL-8 plus anti-IL-8). (C) Anti-IL-8 antibodies used as in B do not affect neutrophil transepithelial migration that occurs spontaneously after surface colonization with *S. typhimurium*. (D) Neutralizing mAb (open bars) against the neutrophil IL-8 receptor (IL-8 receptor A) were only effective at inhibiting transepithelial migration of neutrophils to gradients of IL-8 (500 ng/ml; \* $P < 0.01$ ). In contrast, the same mAb did not prevent transepithelial migration to either transepithelial gradients of fMLP ( $10^{-7}$  M) or *S. typhimurium* stimulation. Data are expressed as mean and SD of triplicate samples for each condition tested and represent one out of two experiments performed with similar results. The y-axis of this figure represents transepithelial migration as defined by PMN migration across an intact epithelial monolayer.

rium induces basolateral secretion of IL-8 (Fig. 2 A). We extended previous suggestions (McCormick et al., 1993) that basolateral IL-8 secretion does not account for the final event of transepithelial migration of neutrophils in response to surface-attached pathogens. As shown in Fig. 2 B, IL-8-dependent transepithelial migration of neutrophils elicited by imposing transepithelial gradients of recombinant human IL-8 is effectively inhibited by neutralizing antibodies to IL-8. In contrast, transepithelial migration across *S. typhimurium*-colonized monolayers is not influenced by the presence of neutralizing IL-8 antibody (Fig. 2 C). Similarly, neutrophil transepithelial migration induced by imposed gradients of IL-8 is also effectively inhibited by blocking antibodies to the IL-8 receptor (IL-8 receptor A), while such antibodies are ineffective at inhibiting transepithelial migration of neutrophils induced by apically attached *S. typhimurium* (Fig. 2 D). Such data indicate that if basolateral IL-8 secretion has a biological role in inflammation it is unlikely to be mediation of the final event by which neutrophils arriving in the immediate subepithelial space are guided across the epithelial monolayer.

### Colonization of the Apical Membrane with *S. typhimurium* Imprints a Directional Chemotactic Signal on the Underlying Matrix

We next sought to determine whether apical membrane colonization by *S. typhimurium* could result in the imprinting of a chemotactic signal within the underlying matrix. As shown in Fig. 3, thoroughly washed, epithelial-stripped matrices synthesized by T84 monolayers not colonized by *S. typhimurium* poorly supported spontaneous transmatrix neutrophil migration. However, neutrophil migration across such control matrices was readily driven by imposing max-

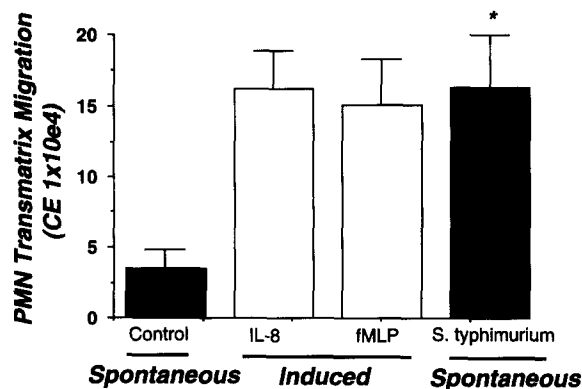
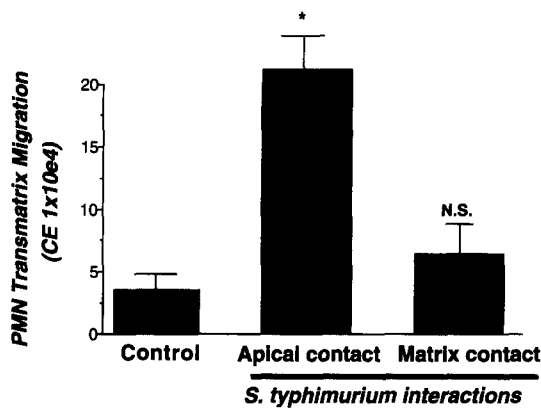


Figure 3. *S. typhimurium* colonization of T84 monolayers imprints underlying matrices with chemotactic signals that drive transmatrix neutrophil migration as effectively as imposed maximal effective gradients of IL-8 (100 ng/ml) or fMLP ( $10^{-7}$  M) Spontaneous (control), migration across matrices isolated from uncolonized T84 monolayers; Induced, migration across such control matrices in response to imposed (maximally effective) chemotactic gradients; Spontaneous (*S. typhimurium*)-spontaneous migration supported by matrices imprinted by *S. typhimurium*-colonized monolayers ( $*P < 0.05$ ). In these experiments, as in all subsequent figures, the T84 epithelial cell monolayers have been removed to expose matrix components (see Materials and Methods). The y-axis, consistent with all subsequent figures, represents transmatrix migration defined as PMN migration across the matrix filter after the epithelial monolayer has been removed.

imally effective transmatrix gradients of neutrophil chemoattractants, fMLP ( $10^{-7}$ M) or rIL-8 (100 ng/ml). In contrast to control matrices, matrices isolated from T84 monolayers apically colonized with *S. typhimurium* were imprinted by signals which supported spontaneous neutrophil migration responses quantitatively similar to those achievable by imposing maximally effective transmatrix gradients of either fMLP, or rIL-8 on control matrices (Fig. 3). Such matrices had undergone large volume washes sufficient to remove unbound soluble molecules (see Materials and Methods). Analyses of numbers of neutrophils adherent to the matrix also provided evidence of an imprinted signal-driving migration through the matrix. While substantial numbers of neutrophils adhered to but did not migrate across matrices obtained from uncolonized T84 monolayers, the spontaneously driven migration of neutrophils across matrix-imprinted *S. typhimurium*-colonized T84 monolayers resulted in depletion of matrix-adherent neutrophils ( $17.23 \pm 2.11$  vs  $3.21 \pm 0.86$  CE  $\times 10^4$ , respectively). The effect of matrix imprinting by *S. typhimurium*-epithelial interactions was seen throughout the time course of the chemotactic assay (Fig. 4). By 90 min  $\sim 50\%$  of neutrophils interfacing with imprinted matrices had successfully transmigrated ( $50.12 \pm 10.00$  vs  $7.31 \pm 1.10$  CE  $10^4$  for transmatrix migration of neutrophils across *S. typhimurium*-imprinted matrices vs. control, respectively;  $10^6$  neutrophils were added to the basolateral surface of each well) across the matrix filter. Thus, matrix imprinted by monolayers that were surface colonized by *S. typhimurium* support spontaneous transmatrix migration and do so at exceedingly high levels.

We next determined whether apical epithelial contact, or simply exposure of *S. typhimurium* to T84 cell matrix components (which might occur during the procedure used to strip colonized epithelial monolayers from underlying matrices), was essential for induction of neutrophil transmatrix migration. Preexposure of matrices derived from control monolayers to *S. typhimurium* did not elicit spontaneous transmatrix neutrophil migration (Fig. 4). Thus, the observed imprinting could not be explained by incidental contamination of the matrix during the stripping procedure.

The relationship between density of surface-colonized *S. typhimurium* and the efficacy of matrix imprinting was also documented ( $7.25 \pm 1.50$ ,  $40.93 \pm 6.8$ ,  $78.31 \pm 7.20$  CE  $10^4$  for 3, 15, and 50 bacteria/epithelial cell, respectively). Thus, increasing the *S. typhimurium* cell-associated populations from 3:15:50 bacteria/epithelial cell before isolating T84-conditioned cell matrix led to progressive efficiency of matrix imprinting. In paired experiments imprinting matrices with *S. typhimurium*-colonized monolayers led to greater transmigration responses than occurred by imposing maximal fMLP gradients across matrices obtained from uncolonized epithelial monolayers ( $78.31 \pm 7.20$  vs.  $39.76 \pm 8.00$  CE  $10^4$  for *S. typhimurium*-imprinted matrices vs. fMLP-induced gradients across control matrices, respectively). Additionally, the kinetics of *Salmonella*-T84 exposure were characterized to determine the duration of *Salmonella*-epithelial interactions that account for maximal transmatrix signals to neutrophils. An inoculum of *S. typhimurium* producing colonization by 50 bacteria/epithelial cell was placed on the apical membrane



**Figure 4.** The effect of direct exposure of control, unimprinted monolayer-derived matrices to *S. typhimurium*. In contrast to unimprinted matrices that support spontaneous transmatrix migration of neutrophils (*Apical contact*), direct exposure of unimprinted matrices to bacteria (applied at same density and length of time as in colonized monolayers) does not support spontaneous neutrophil migration. Thus the imprinting observed specifically requires a bacterial-epithelial interface ( $*P < 0.025$ ).

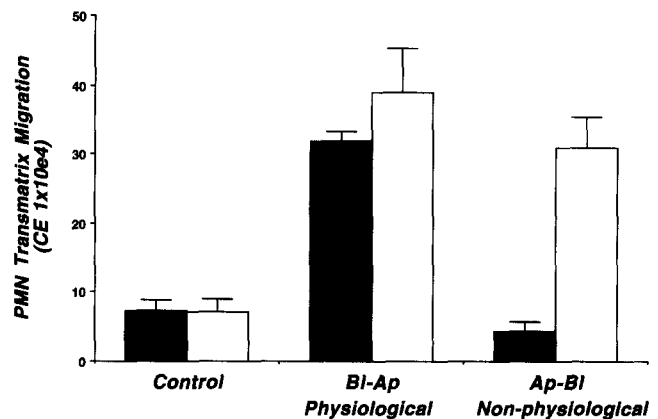
of T84 monolayers grown on permeable supports for periods ranging from 0 to 90 min, before preparing T84-conditioned matrices. Increasing the duration of *S. typhimurium*-epithelial interactions led to a corresponding increase in imprinted transmatrix migration of neutrophils; peaking at 45 min and maintaining a plateau thereafter (data not shown). Further, filter/matrix-associated neutrophil density remained constant throughout the time course of *S. typhimurium* exposure ( $\sim 8.00 \text{ CE } 10^4$ ).

#### **Matrix Imprinting by *S. typhimurium*-colonized Monolayers Is Directional**

To efficiently drive migration of neutrophils exiting the vascular bed to an immediate subepithelial position, matrix imprinting should provide a directional chemotactic gradient. To test whether the imprinted signal was chemotactic or simply chemokinetic, we examined neutrophil migration across imprinted matrices in apical-to-basal and basal-to-apical directions. As shown in Fig. 5, the chemokine signal imprinted on matrices conditioned by *S. typhimurium*-colonized monolayers supported basolateral-to-apical, but not apical-to-basolateral migration. However, imposed gradients of fMLP ( $10^{-7} \text{ M}$ ) were shown to effectively drive neutrophil migration in either direction across the isolated matrix.

#### **Effect of Bacterial and Epithelial Protein Synthesis on Matrix Imprinting**

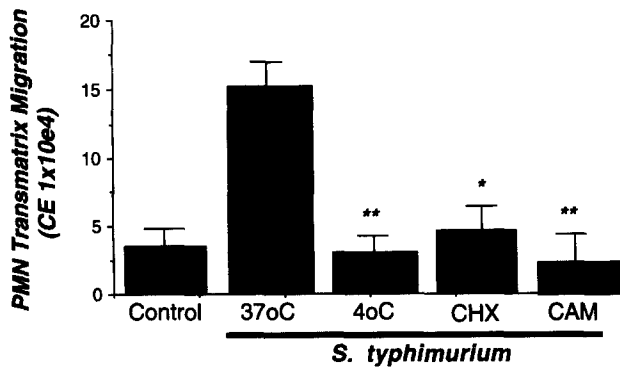
To address whether metabolic events after bacterial-epithelial contact are, in part, responsible for imprinting the matrix with a chemotactic signal after surface colonization by *S. typhimurium*, imprinting was performed at  $4^\circ\text{C}$ , a temperature at which bacterial-epithelial adhesive associations can still occur (Behlau and Miller, 1993). As shown in Fig. 6 and in contrast to matrices imprinted at  $37^\circ\text{C}$ , matrices imprinted at  $4^\circ\text{C}$  did not support subsequent neutrophil migration (neutrophil migration assayed after shifting isolated matrix to  $37^\circ\text{C}$ ).



**Figure 5.** Matrix imprinting by *S. typhimurium*-colonized monolayers supports chemotactic (directional) neutrophil migration. To assess whether the imprinted signal was chemotactic or chemokinetic, neutrophil migration across imprinted matrices was performed in both physiologic (tissue-to-lumen) and non-physiologic (lumen-to-tissue) directions. As shown, neutrophil transmigration was supported only in the physiologic (tissue-to-lumen) orientation ( $*P < 0.01$ ). In the nonphysiologic orientation, T84 epithelial monolayers were plated in the conventional manner such that the apical surface interfaced with the upper reservoir (Parkos et al., 1991, 1992). Under such conditions, matrices were isolated from *S. typhimurium*-infected cell monolayers as described in detail (see Materials and Methods). However, in such an orientation neutrophils were added to the nonphysiologic surface (apical) which interfaced with the upper reservoir. These conditions are comparable to the physiological conditions since comparison of basal transmatrix migration in response to imposed external fMLP gradients shows no difference as a function of polarity, unlike IL-8-imprinted gradients. ■, *Salmonella*-imprinted matrices; □, fMLP-induced gradients.

We next determined whether bacterial and/or epithelial protein synthesis were required after bacterial-epithelial contact in order to successfully imprint underlying matrices. *S. typhimurium* were exposed to chloramphenicol ( $100 \mu\text{g/ml}$ ), a bacteriostatic bacterial-specific protein synthesis inhibitor (see Materials and Methods), just before and during the incubation period in which matrices are imprinted. As shown in Fig. 6, the neutrophil transmatrix migration response elicited by chloramphenicol-treated *S. typhimurium*-conditioned matrix was 85% inhibited compared with that occurring across matrix imprinted in the absence of chloramphenicol. In previous experiments, quantitation of colonized epithelial lysates revealed that such chloramphenicol exposure did not substantially affect the ability of *S. typhimurium* to adhere to T84 monolayers, nor did chloramphenicol exposure measured over 3 h substantially affect the T84 transepithelial resistance (McCormick et al., 1993). Such data suggest that bacterial-epithelial apical membrane contacts and subsequent bacterial protein synthesis once contacts are established are crucial to subsequent generation of a transmatrix signaling response to neutrophils.

Matrix imprinting was likewise assessed in the presence of the eucaryotic protein synthesis inhibitor, cycloheximide at a concentration that inhibits  $>80\%$  of protein synthesis, a condition previously shown not to impair epithelial barrier function nor restrict *S. typhimurium* attachment



**Figure 6.** Matrix imprinting requires cooperative metabolic events between bacteria and epithelial cells. Surface colonization by *S. typhimurium* at 37°C (normal colonization conditions) is required for imprinting to occur, since a shift in temperature to 4°C inhibits matrix imprinting by *S. typhimurium*. Additionally, selective inhibition of either prokaryote protein synthesis by chloramphenicol (CAM, 100 µg/ml) or eucaryotic protein synthesis by cycloheximide (CHX, 2 µg/ml) effectively blocks matrix imprinting (\* $P < 0.05$ ; \*\* $P < 0.025$ ).

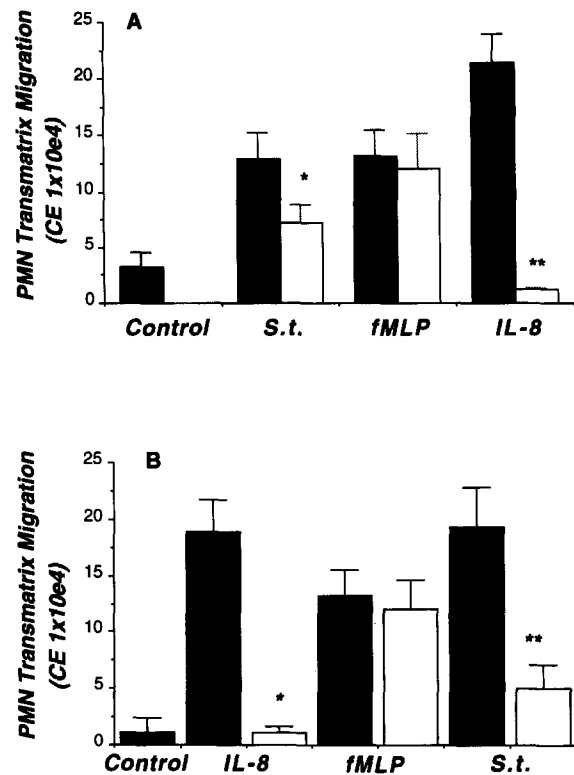
to the epithelium (McCormick et al., 1993). As shown in Fig. 6, eucaryotic protein synthesis after *S. typhimurium* binding was also required to successfully imprint matrices.

### IL-8 Is a Major Component of Salmonella-Epithelial Imprinting of Cell Matrix

We next examined whether the polarized IL-8 secretion observed which follows *S. typhimurium* attachment to epithelial cells was, in part, responsible for the observed imprinting of underlying matrices. As shown in Fig. 7 A, neutralizing polyclonal antibodies to IL-8 (30 µg/ml) have no effect on migration of neutrophils across matrices obtained from uncolonized T84 monolayers in response to formyl peptide gradients, but effectively inhibit neutrophil migration across such matrices driven by imposed gradients of rIL-8 (100 ng/ml). Moreover, neutralizing antibodies to IL-8 effectively reduced neutrophil transmatrix migration across *Salmonella*-conditioned matrices by ~50% ( $12.30 \pm 2.40$  vs.  $7.231 \pm 1.60$  CE  $10^4$  for *Salmonella*-conditioned T84 cell matrix in the presence and the absence of 30 µg/ml neutralizing polyclonal IL-8 antibodies).

### Spontaneous Chemotaxis of Neutrophils across Imprinted Matrices is Directed by the IL-8 Receptor

The involvement of IL-8 in matrix imprinting was further characterized by examining the effects of inhibiting antibodies to the IL-8A receptor on chemotaxis through imprinted matrices. As shown in Fig. 7 B, mAbs to the IL-8 receptor (IL-8 receptor A) present on neutrophils do not inhibit transmatrix migration of neutrophils in response to fMLP gradients ( $10^{-7}$  M), but ablate neutrophil transmatrix migration to imposed transmatrix gradients of IL-8 (100 ng/ml) (95% inhibited in the presence of 50 µg/ml IL-8 receptor mAb). Spontaneous neutrophil migration across imprinted matrices was inhibited by ~70% by this IL-8 receptor antibody. Together, these data indicate that the chemotactic signal imprinted on the matrix after *S. typhi-*



**Figure 7.** The effect of neutralizing anti-IL-8 on matrix imprinting, as well as the effect of anti-IL-8 high-affinity receptor antibody on neutrophil migration across T84-derived matrices. (A) In contrast to control unimprinted matrices, matrices either imprinted by *S. typhimurium* (S.t.) or matrix across which fMLP or IL-8 gradients are imposed support substantial neutrophil migration (solid bars; absence of IL-8 antibody). IL-8-neutralizing antibody (50 µg/ml) (open bars), inhibits ~50% (\* $P < 0.10$ ) of spontaneous migration across imprinted matrices. As a negative control, IL-8 antibody has no effect on migration across control matrices driven by exogenous fMLP ( $10^{-7}$  M). As a positive control, IL-8 antibody ablates migration across control matrices driven by exogenous IL-8 (100 ng/ml) gradients (\* $P < 0.01$ ). (B) Control, rIL-8 (100 ng/ml), and fMLP ( $10^{-7}$  M) correspond to unimprinted matrices across which specific gradients were imposed. S.t., matrices imprinted by *S. typhimurium* interactions. Neutrophil migration in the absence of (solid bars) or presence of (open bars) blocking antibody to the IL-8 receptor (IL-8 receptor A) (50 µg/ml) is shown. The majority of the imprinted signal for transmatrix migration appears neutralized by the antibody, whereas this antibody has no effect on migration which occurs via another neutrophil chemotactic receptor (fMLP) (\* $P < 0.01$ ; \*\* $P < 0.025$ ).

*murium*-epithelial colonization is largely due to IL-8 and can also be detected by the IL-8 receptor.

### Induction of IL-8 Imprinting in Control Matrices

The ability of IL-8 to imprint chemotactic memory on matrices generated by T84 monolayers was demonstrated by direct conditioning of control matrices with IL-8. Control matrices were exposed to rIL-8 gradients (apical > basolateral) of 0, 2, 20, and 200 ng/ml for 45–90 min. As shown in Table I, exogenous IL-8 gradients produced time-dependent matrix imprinting. The largest signal was seen at 90 min after imprinting in a 200 ng/ml gradient and the im-

**Table I. Exogenous Conditioning of Control Matrices with IL-8 and the Ability to Imprint Chemotactic Signals**

Time	[IL-8]	Minus IL-8 Ab*	Plus IL-8 Ab*
(min)	(ng/mL)		
45	0	2.651 ± 0.984	1.721 ± 0.365
	200	3.714 ± 1.231	1.956 ± 0.841 <sup>‡</sup>
90	0	2.560 ± 0.958	1.512 ± 0.365
	200	11.325 ± 2.65	1.856 ± 0.951 <sup>§</sup>

Data represent mean of triplicate samples ± SD. One representative experiment of two performed (all showing the same result).

\*Values represent the number of neutrophils (CE × 10<sup>4</sup>) that have migrated completely across filter matrices in response to exogenous conditioning with 200 ng/ml IL-8 for either 45 or 90 min (see Materials and Methods). Neutrophils are quantitated based on known PMN standard using the myeloperoxidase assay (see Materials and Methods). IL-8 at 2 and 20 ng/ml did not significantly imprint matrices such that transmatrix migration was enhanced (not shown).

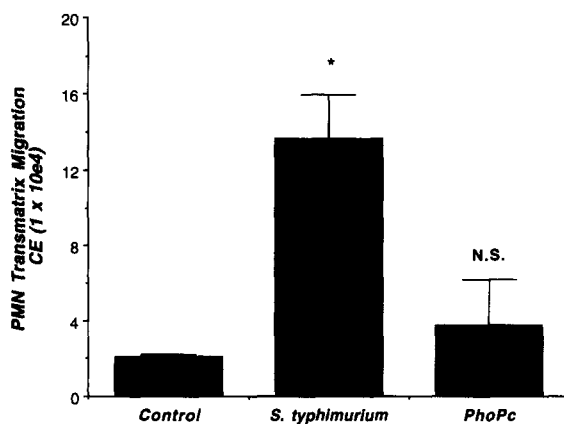
<sup>‡</sup>(*P* < 0.100)

<sup>§</sup>(*P* < 0.025)

printed gradient (i.e., retained after multiple high volume washes) was effectively inhibited by IL-8 antibodies (~90%).

### *S. typhimurium* Mutant PhoP<sup>c</sup> Fails to Induce IL-8 Secretion and Matrix Imprinting

*S. typhimurium* PhoP<sup>c</sup> (strain CS022) carries a constitutive mutation within the *S. typhimurium* *PhoP* regulatory locus (phenotype PhoP<sup>c</sup>) that results in attenuated host virulence in mice (Miller and Mekalanos, 1990; Miller, 1991; Behlau and Miller, 1993). Unlike wild-type *S. typhimurium*, the mutant PhoP<sup>c</sup> did not induce T84 cell-polarized monolayers to secrete IL-8 (2.63 ± 1.00 vs. 0.200 ± 0.151 ng/ml secreted into the basolateral compartment for wild-type *S. typhimurium* 14028s and PhoP<sup>c</sup>, respectively; *P* < 0.05). As shown in Fig. 8, the mutant PhoP<sup>c</sup> also was un-



**Figure 8.** *S. typhimurium* mutant PhoP<sup>c</sup> is attenuated in its ability to drive spontaneous PMN transmatrix migration. Matrices derived from uncolonized monolayers or from monolayers with the same surface-attached densities of either wild-type *S. typhimurium* or the invasion defective mutant *S. typhimurium* PhoP<sup>c</sup> were studied. The PhoP<sup>c</sup> mutant display limited virulence in mice (Miller and Mekalanos, 1990) and do not result in basolateral IL-8 secretion (see text). As shown, *S. typhimurium* mutant PhoP<sup>c</sup> poorly imprints T84 cell matrices compared with wild-type *S. typhimurium* (\**P* < 0.01).

able to induce matrix imprinting of chemotactic signals even though PhoP<sup>c</sup> adhered to T84 apical epithelial surfaces (McCormick et al., 1995).

### Discussion

Attachment of *S. typhimurium* to the apical membrane of intestinal epithelial cells elicits directed neutrophil migration from the vasculature, across the subepithelial matrix and ultimately across the epithelium itself (Takeuchi, 1967; Day et al., 1978). Remarkably these events occur while the epithelium is still intact (Takeuchi, 1967; Day et al., 1978). While the mechanisms driving these responses have only recently attracted attention, there is now substantial evidence that the epithelial cells themselves may play a proactive role in organizing/initiating such inflammatory responses (Agace et al., 1993; Eckmann et al., 1993; McCormick et al., 1993; Jung et al., 1995). It is clear that bacterial binding to epithelial cells can influence production of important regulators of inflammation. For example, IL-6 production is elicited by the binding of adherent *Escherichia coli* to bladder or kidney epithelial cells (Hedges et al., 1991), and we (McCormick et al., 1993) and others (Eckmann et al., 1993) have shown that *S. typhimurium* interaction with the apical pole of intestinal epithelial cells elicits secretion of bioactive cytokines. Importantly, the pattern of secretion of chemokines shown by T84 cells in response to challenge accurately portrays the response pattern observed with freshly isolated human colonocytes (Jung et al., 1995).

Here we show that biophysically confluent T84 cell monolayers apically colonized by *S. typhimurium* secrete signals that imprint chemotactic memory on underlying matrices. Antibody neutralization experiments using either polyclonal antibody directed at IL-8 itself or monoclonal antibody against the IL-8 receptor on neutrophils, indicate that a bulk of this imprinted bioactivity can be attributed to epithelial basolateral secretion of IL-8 stimulated by *S. typhimurium* contact with the apical membrane. Experiments using such neutralizing IL-8 and blocking IL-8A receptor antibodies in the presence of intact epithelial monolayers suggest that although basolateral IL-8 secretion is key to imprinting signals chemotactic for neutrophils on subepithelial matrices, IL-8 secretion is not needed to drive the final step of migration of subepithelial neutrophils to the luminal compartment. Given that basolateral secretion of IL-8 would not result in a favorable transepithelial gradient to drive transepithelial migration, this finding is not surprising. Furthermore, such observations are consistent with recent findings that an apically secreted chemokine with physical characteristics unlike IL-8 appears responsible for driving this final step of transepithelial migration (McCormick, B. A., and J. L. Madara, manuscript in preparation). These data suggest that a primary role for basolateral secretion of IL-8 by epithelia is recruitment of neutrophils through the matrix to the subepithelial space. Such events may have substantial importance in assisting movement of neutrophils to the subepithelial space. For example, it has been noted in patients with cystic fibrosis that neutrophil elastase can induce IL-8 gene expression in respiratory epithelia (Nakamura et al., 1992). Additionally, inhibition of IL-8 gene



expression by aerosolized secretory leukoprotease inhibitor suppresses both IL-8 secretion and neutrophil infiltration of this epithelium in this disorder (McElvaney et al., 1992). Although our functional data strongly imply the existence of an IL-8 gradient in the matrix after *Salmonella* colonization, we have not been able to document the existence of this gradient by direct physical means.

Known distorting effects of solvent flow in the microenvironment of the lamina propria (Jodal and Lundgren, 1986) would seem to require long range matrix binding of chemotactic signals. Epithelial-derived IL-8 exhibits many properties that would make this chemokine well-suited for this purpose. IL-8 is extremely resistant to inactivation (both in vitro and in vivo) (Colditz et al., 1989). Thus, once present in inflamed tissue IL-8 is likely to retain its biological activity for several hours, as shown by local intradermal administration in animals and humans (Colditz et al., 1989; Leonard et al., 1991; Swensson et al., 1991). In contrast to IL-8, chemokines such as fMLP or LTB<sub>4</sub> are degraded rapidly by oxidation or hydrolysis (Baggiolini et al., 1994). Most importantly for sites like the subepithelial matrix of intestinal mucosa where volume flow is extremely high, IL-8, due to its highly cationic nature, binds avidly to glycosaminoglycans of the tissue matrix (Baggiolini et al., 1992), thus making such bound IL-8 gradients particularly resistant to sweeping away effects of fluid flow. Such binding characteristics of IL-8 are further highlighted by the ability of this cytokine to provide a surface-fixed gradient capable of sustaining haptotactic migration of A 2058 melanoma cells (Wang et al., 1990). The IL-8 receptor antibody and the IL-8 neutralizing antibody were both highly effective at blocking migration induced by exogenously added human recombinant (hr)IL-8. However only ~50–60% of the matrix imprinted signal was neutralized by the blocking antibody, while the IL-8 receptor antibody was more effective (~70–80% inhibition). These observations leave open the possibility that other imprinting signals may contribute to this response, perhaps including other signals which are agonists for the IL-8 receptor. Alternatively, such observed minor differences in the efficiency of inhibition by these antibodies may have trivial explanations relating to the complex geometry of the interactions taking place in the matrix. In addition, when neutrophil migration across complex biological matrices is driven by imprinted IL-8 gradients, it is not clear which of the two recently characterized IL-8 receptors would be used. Our data obtained using a blocking antibody with specificity (Chuntharapai et al., 1994) for the IL-8 A receptor on neutrophils suggest that this receptor is sufficient to drive such complex responses. We, however, cannot rule out the possibility that both the high- or the low-affinity receptor might have modulating influences on IL-8 responses in such complex biological situations. Not only is *S. typhimurium* attachment to the apical membrane required to initiate matrix imprinting, but reciprocal metabolic interactions are required as well. In particular, we found that inhibition of postbacteria-epithelial-binding metabolic events or inhibition of protein synthesis in either the bacteria or the epithelial cells (after binding) led to significantly attenuated matrix imprinting. Such observations suggest that bacterial gene products newly expressed as a result of epithelial binding lead to reciprocal

alteration in protein expression by the epithelial cells. This idea is consistent with the requirement for inducibly expressed virulence factors necessary for *Salmonella* pathogenesis (Lee and Falkow, 1990; Alpuche Aranda et al., 1992; McBeth and Lee, 1993; Pegues et al., 1995). 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 Pegues (1995) suggest that the PhoP/PhoQ regulon regulates extracellular transport of proteins by transcriptional repression of secretion determinants and that such secreted proteins may be involved in signaling epithelial cells to endocytose bacteria. The PhoPc mutant strain of *S. typhimurium* carries a mutation within the *phoP* regulatory locus that results in attenuated host virulence for mice (Miller and Mekalanos, 1990). Here we show that, while this strain is able to attach to epithelial cells, it does not lead to basolateral IL-8 secretion, and imprinting of the underlying matrix does not occur. Such observations suggest that matrix imprinting, such as that described here, may be an important signaling event in response to eucaryotic-procaryotic interactions.

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