

Requirement of the *Shigella flexneri* Virulence Plasmid in the Ability To Induce Trafficking of Neutrophils across Polarized Monolayers of the Intestinal Epithelium

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Attachment of an array of enteric pathogens to epithelial surfaces is accompanied by recruitment of polymorphonuclear leukocytes (PMN) across the intestinal epithelium. In this report, we examine how *Shigella*-intestinal epithelium interactions evoke the mucosal inflammatory response. We modeled these interactions in vitro by using polarized monolayers of the human intestinal epithelial cell line, T84, isolated human PMNs, and *Shigella flexneri*. We show that *Shigella* attachment to T84-cell basolateral membranes was a necessary component in the signaling cascade for induction of basolateral-to-apical directed transepithelial PMN migration, the direction of PMN transepithelial migration in vivo. In contrast, attachment of *Shigella* to the T84-cell apical membrane failed to stimulate a directed PMN transepithelial migration response. Importantly, the ability of *Shigella* to induce PMN migration across epithelial monolayers was dependent on the presence of the 220-kb virulence plasmid. Moreover, examination of *Shigella* genes necessary to signal subepithelial neutrophils established the requirement of a functional type III secretion system. Our results indicate that the ability of *Shigella* to elicit transepithelial signaling to neutrophils from the basolateral membrane of epithelial cells represents a mechanism involved in *Shigella*-elicited enteritis in humans.

The intestinal mucosa is routinely exposed to a wide range of microorganisms and foreign substances and provides both a physical and an immunological barrier to such challenges from the exterior environment. Epithelial cells in the gastrointestinal tract were classically thought to serve the dual purpose of regulating salt, water, and nutrient transport and of providing a barrier to passive diffusion of molecules between the intestinal lumen and tissue space (9, 14). However, it is becoming clear that interactions between intestinal epithelial cells and bacteria may play a role in orchestrating the inflammatory response. For example, attachment of an array of bacterial pathogens, including *Salmonella* and *Shigella*, to epithelial cell surfaces is accompanied by recruitment of host defense cells exhibited by transepithelial migration of polymorphonuclear leukocytes (PMN) in the basolateral-to-apical direction. Such transmigration of PMN across intestinal epithelia represents the histological definition of acute intestinal inflammation and is a hallmark of bacterial enterocolitis caused by enteric pathogens such as *Salmonella* (8, 20, 31, 44, 52) and *Shigella* (5).

The details of how such host-pathogen interactions evoke the classical histological lesion of PMN transepithelial migration are currently being studied. It is becoming increasingly clear that *Salmonella*-intestinal apical epithelial cell contacts result in the generation of a signaling cascade which directs the trafficking of PMN in the basolateral-to-apical direction (28–30). *Salmonella typhimurium* induces the intestinal epithelium to secrete a repertoire of chemokines which play an active role in

recruiting PMN from the peripheral circulation and directing them across the epithelium to the intestinal lumen (28–30). Such epithelial orchestration of PMN movement is thought to be mediated by polarized secretion of distinct chemokines and neutrophil chemoattractants (29, 30). Interleukin-8, for example, is secreted basolaterally by intestinal epithelial cell lines in vitro (10, 29, 30), as well as in the human colon (19), in response to either proinflammatory cytokines or invasive bacteria. Such secretion leads to gradients of interleukin-8 being formed in the subepithelial extracellular matrix and is largely responsible for the movement of PMN through the extracellular matrices of model epithelia (28). However, to establish gradients which would direct PMN to migrate across the epithelium to the apical surface, these chemokines must be preferentially secreted apically. Recently, the first such chemokine has been described: pathogen-elicited epithelial chemoattractant (30).

The mechanisms which underlie PMN transepithelial migration induced by *Shigella*-host interactions are not as well characterized, owing, at least in part, to the requirement of *Shigella* entry into the basolateral domain of the intestinal epithelium. A recent investigation by Perdomo et al. described the ability of *S. flexneri* to induce PMN transmigration through a confluent epithelial cell monolayer, with the implication being that transmigrating PMN play an active role at early stages of epithelial cell invasion by opening the paracellular pathway for bacterial entry into differentiating colonocytes (42). However, a paradoxical result of their study was the observation that wild-type and avirulent (noninvasive) *S. flexneri* strains demonstrated equal abilities to induce PMN transepithelial migration across intestinal epithelial cell monolayers.

In the present study, we took a different approach and asked whether positioning *Shigella* adjacent to the basolateral epithe-

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TABLE 1. Bacterial strains used in this study and their relevant virulence properties

| Strain ^a | Description | Invasion of HeLa cells | Type III secretion | Reference |
|---------------------|---|------------------------|--------------------|-----------|
| 2457T | Wild type | + | + | 12 |
| 2457O | 2457T <i>virF</i> ::IS2 | - | - | 34 |
| BS103 | 2457T cured of the virulence plasmid | - | - | 27 |
| BS228 | 2457T <i>ipaB</i> :: Δ plac Mu53 | - | + ^b | 18 |
| BS232 | 2457T <i>mxi</i> :: <i>lacZ</i> | - | - | 18 |
| BS545 | 2457T <i>spa33</i> :: <i>aphA3</i> | - | - | 49 |
| BS547 | 2457T <i>mxiM</i> :: <i>aphA3</i> | - | - | 50 |
| M90T | Wild type | + | + | 47 |
| BS176 | M90T cured of the virulence plasmid | - | - | 48 |

^a All strains are derivatives of *S. flexneri* 2a except M90T and BS176, which are strains of *S. flexneri* 5.

^b Insertion in BS228 results in synthesis of a truncated IpaB product and is polar on the downstream *ipa* genes; i.e., IpaC, IpaD, and IpaA are not synthesized.

lial membrane domain of their target tissue (i.e., the intestinal epithelial surface through which *Shigella* invades) promoted the generation of discrete signals necessary to evoke directed migration of PMN across the intestinal monolayer in the basolateral-to-apical direction, analogous to the direction of PMN movement across the intestinal epithelium during active states of inflammation. These studies were performed with *Shigella flexneri* strains and human peripheral blood PMN in association with polarized monolayers of the human-derived, physiologically confluent, crypt-like cell line T84 to model pathogen-induced intestinal inflammation. We report that model intestinal epithelia respond to basolateral membrane-*Shigella* interactions by promoting signals essential to drive PMN transepithelial migration in the biologically relevant basolateral-to-apical direction. This signaling response exhibited a strict dependence on *Shigella* contact with the epithelial basolateral membrane domain, required genes present on the 220-kb large virulence plasmid, and was dependent upon a functional *Shigella* type III secretion apparatus.

MATERIALS AND METHODS

Cell culture. T84 intestinal epithelial cells (passages 70 to 95) were grown in a 1:1 mixture of Dulbecco-Vogt-modified Eagle medium and Ham's F-12 medium supplemented with 15 mM HEPES buffer (pH 7.5), 14 mM NaHCO₃, 40 mg of penicillin per ml, 8 mg of ampicillin per ml, 90 mg of streptomycin per ml, and 5% newborn calf serum (22, 36, 39, 40). Monolayers were grown on 0.33-cm² suspended collagen-coated permeable polycarbonate filters (Costar Corp., Cambridge, Mass.) and used 7 to 14 days after plating, as described previously (22, 39). A steady-state transepithelial cell resistance, approximately 1,500 ohm · cm², is reached in 5 days, with variability largely related to cell passage number. Monolayers received one weekly feeding following initial plating. Inverted monolayers used to study the transmigration of neutrophils in the physiological basolateral-to-apical direction were constructed as described previously (22, 36, 39, 40).

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1.

Bacteria were routinely grown at 37°C in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.). A 100- μ l volume of a stationary-phase culture was used to inoculate 10 ml of TSB, and the bacteria were grown in a shaking incubator for approximately 2 h at 37°C to the mid-exponential phase of growth (optical density at 600 nm of 0.30). Tryptic soy agar is TSB containing 12 g of Bacto Agar (Difco) per liter and 0.025% Congo Red (Sigma Chemical Co., St. Louis, Mo.).

Electrical measurements. To assess currents, transepithelial potentials, and resistance, a commercial voltage clamp (Bioengineering Department, University of Iowa) was used and interfaced with an equilibrated pair of calomel electrodes submerged in saturated KCl along with a pair of Ag-AgCl electrodes submerged in Hanks balanced salt solution containing Ca²⁺ and Mg²⁺ [HBSS(+)]. Agar bridges were used to interface the electrode with the solution on either side of

the monolayers (one calomel and one Ag-AgCl electrode in each well), and the short-circuit current and resistance were measured as detailed elsewhere (24).

***Shigella* invasion into T84 intestinal epithelial cell monolayers.** T84 monolayers were infected by the method of McCormick with slight modifications (29). Inverted monolayers were drained of media and gently washed with HBSS(+) containing 10 mM HEPES (pH 7.4; Sigma). Bacterial samples representing an inoculation ratio of 20 bacteria/epithelial cell were added to the basolateral side of the monolayer, and bacterial invasion was assessed after incubation at 37°C for 90 min. Cell-associated bacteria included populations of bacteria adherent to and/or internalized into the T84 monolayers and were released by incubation with 1% Triton X-100 (Sigma). Internalized bacteria were those obtained from lysis of the epithelial cells with 1% Triton X-100 after the addition of gentamicin (50 μ g/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. For both cell-associated and internalized bacteria, 0.9 ml of Luria-Bertani broth was then added and each sample was vigorously mixed and quantitated by plating for CFU on MacConkey agar medium.

PMN transepithelial migration assay. The PMN transepithelial migration assay has been detailed previously (16, 39, 40). Human PMN were purified from whole blood (anticoagulated with 13.2 g of citrate and 11.2 g of dextrose in 500 ml of water [pH 6.5]) collected by venipuncture from normal human volunteers of both sexes. The buffy coat was obtained by centrifugation at 400 \times g at room temperature. Plasma and mononuclear cells were removed by aspiration, and the majority of erythrocytes were removed by a 2% gelatin sedimentation technique as described previously (40). Residual erythrocytes were then removed by gentle lysis in cold NH₄Cl lysis buffer. This technique allowed the rapid isolation (90 min) of functionally active PMN that were 95% pure with 98% viability as determined by trypan blue exclusion. After isolation, PMN were suspended in modified HBSS (without Ca²⁺ and Mg²⁺ but with 10 mM HEPES [pH 7.4]) at 4°C at a concentration of 5 \times 10⁷/ml and were used for experiments within 1 h after isolation.

Shigella-epithelial cell-PMN interactions are depicted in Fig. 1. Briefly, before the addition of PMN to this assay system, confluent, inverted T84 polarized monolayers (3.5 \times 10⁵ cells/well) (22, 40) were rinsed extensively in HBSS(+) to remove residual serum components. *Shigella* strains were prepared by washing the bacteria twice in HBSS(+) and resuspending them in 300 μ l of buffer per 10 ml of culture (final bacterial concentration, approximately 1.5 \times 10⁹ bacteria per ml). For basolateral surface exposure, 25 μ l of the bacterial suspension (3.5 \times 10⁷ bacteria) was directly added to the upper compartment of inverted T84 monolayers, at a multiplicity of infection of 100 bacteria/epithelial cell, after removal of the basolateral buffer. In studies requiring apical surface exposure, inverted monolayers were removed from each well and placed in a moist chamber such that the epithelial apical membrane (lower compartment) was oriented upward. Again, 25 μ l of the bacterial suspension was gently distributed onto the apical surface. For simplicity, the reservoir is referred to by the epithelial membrane domain with which it interfaces (i.e., apical or basolateral). *Shigella* strains were incubated at either the basolateral or apical epithelial interface for 90 min at 37°C. Nonadherent bacteria were next removed by washing three times in HBSS(+) buffer, and under these conditions it was determined that there were 80 cell-associated bacteria/epithelial cell. The monolayers were then transferred into fresh 24-well tissue culture trays containing 1.0 ml of HBSS buffer in the bottom (apical) compartment and 140 μ l in the top (basolateral) compartment. To the basolateral bath, 40 μ l of isolated PMN (10⁶ cells) was added to each monolayer and incubated for 150 min at 37°C. Positive control transmigration assays were performed by the addition of chemoattractant (1 μ M *N*-formylmethionylleucyl phenylalanine [fMLP]; Sigma) to the opposing apical reservoir. All experiments were performed in a room whose temperature was 37°C to ensure

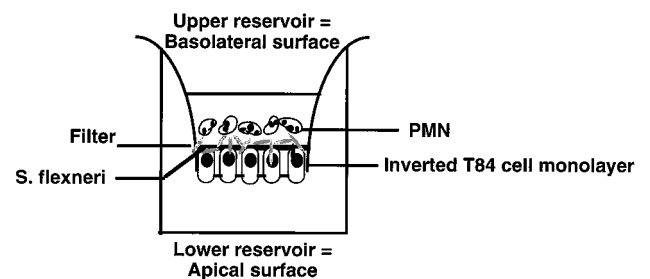


FIG. 1. *Shigella*-induced PMN transepithelial migration assay. In this assay, the basolateral surface of inverted T84 monolayers are first colonized by *S. flexneri* and then washed so that only the cell-associated population remains (cell-adherent plus cell-internalized bacteria). PMN are then placed in the upper (basolateral) reservoir, where they come in contact with the basolateral surface and are subsequently judged for their ability to migrate across these inverted T84 monolayers.

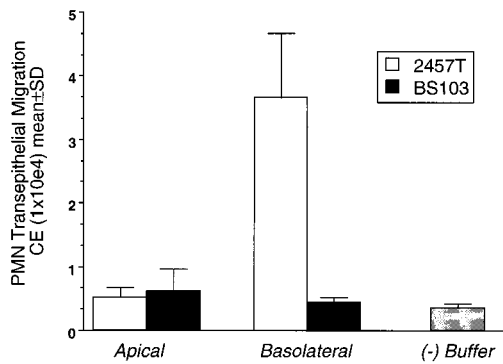


FIG. 2. *S. flexneri*-T84-cell basolateral membrane association induces PMN trans epithelial migration. Polarized monolayers of T84 intestinal epithelial cells were either apically or basolaterally exposed to wild-type *S. flexneri* 2457T (open bars) or a noninvasive plasmid-cured strain BS103 (solid bars) at a density of 80 cell-associated bacteria/epithelial cell. The ability of *S. flexneri* to induce PMN trans epithelial migration was assessed 90 min later (see Materials and Methods). The negative control (-) (gray bar) represents HBSS(+) buffer in the absence of bacteria or a chemotactic stimulus. A positive control was established by using imposed gradients to the chemotactic peptide fMLP (10^{-7} M) [15.12 ± 2.36] $\times 10^4$ CE). Data are means and SD for four monolayers in a single experiment and are representative of four separate experiments.

that the epithelial monolayers, solutions, and plastic ware were maintained at a uniform temperature.

Transmigration was quantified by assaying for the PMN azurophilic granule marker myeloperoxidase, as described previously (22, 39, 40). After each transmigration assay, PMN cell equivalents (CE) were assessed as the number of PMN that had completely traversed the monolayer (i.e., moved into the apical reservoir). Since variation exists in trans epithelial cell resistance between groups of monolayers (baseline resistance, 650 to 1,500 $\text{ohm} \cdot \text{cm}^2$) and in PMN obtained from different donors, individual experiments were performed with large numbers of monolayers and PMN obtained from single blood donors. PMN isolation was restricted to 10 different donors (repetitive donations) over the course of these studies.

Preparation of *S. flexneri* culture supernatants. Aerobically grown cultures of *S. flexneri* at 37°C in TSB (prepared as above) were washed twice in HBSS(+) and resuspended to a final concentration of approximately 5×10^8 cells/ml. After an incubation for 1.5 h at 37°C, the suspensions were centrifuged free of bacteria ($6,000 \times g$ for 10 min) and the *Shigella* conditioned buffer supernatant was collected and passed through a 0.2- μm -pore-size filter. The supernatant was then applied to the basolateral surface of epithelial cell monolayers and assessed for the ability to induce PMN trans epithelial migration.

Presentation of data. PMN transmigration results are represented as PMN CE derived from a daily standard PMN dilution curve. Reservoir-associated PMN (i.e., PMN which had completely traversed the monolayer) are represented as the number of PMN CE per milliliter (total volume, 1 ml). Values are expressed as means and standard deviation (SD) of individual experiments done in triplicate n times. *Shigella* invasion and myeloperoxidase assay data were compared by Student's t test.

RESULTS

Basolateral colonization by *S. flexneri* initiates basolateral-to-apical directed trans epithelial migration of PMN. *S. flexneri* was initially examined for its ability to adhere to and be internalized by polarized T84 intestinal epithelial cell monolayers. An experiment in which an original inoculum of 20 bacteria/epithelial cell was placed on either the apical or basolateral membrane of polarized T84-cell monolayers for 90 min demonstrated that *S. flexneri* preferentially entered epithelial cells via the basolateral membrane domain ($1.23\% \pm 0.11\%$ and $0.087\% \pm 0.004\%$ of the original inoculum was internalized for basolateral and apical membrane association, respectively, of wild-type strain 2457T). These results confirm those of previous investigators (35, 41). The effects of such bacterium-epithelial cell interactions on T84 trans epithelial cell resistance were also determined. Trans epithelial cell resistance to passive ion flow is an extremely sensitive measure of barrier function in

high-resistance epithelia such as T84 monolayers (21). Due to the asymptotic flux-resistance relationship, perturbations so minimal that they induce barely detectable increases in the trans epithelial flux of inert solutes routinely elicit sizable decrements in resistance in these high-resistance monolayers (15, 23). To gain insight into the epithelial barrier function integrity during *S. flexneri* colonization, we investigated the stability of trans epithelial cell resistances over time. Trans epithelial cell resistance at a final colonization density of 80 cell-associated bacteria/epithelial cell remained high throughout a 4-h colonization period at 37°C. They were observed to fall only after 4 h of bacterial colonization, which also corresponded to a similar fall in resistance from control noncolonized epithelial monolayers. However, physiological confluency was maintained even under these conditions (i.e., resistances in substantial excess of $250 \text{ ohm} \cdot \text{cm}^2$ [15, 23]). Thus, since the colonized monolayers maintained an appropriate barrier function throughout a 4-h time course, these specific conditions utilizing 7- to 9-day-old monolayers were used for all neutrophil experiments reported below.

Since recent evidence (35, 41) and our own results (see above) strongly suggest that *S. flexneri* preferentially enters the intestinal epithelia via the basolateral membrane domain, we investigated whether such basolateral exposure by *S. flexneri* was a key requirement for induction of the signaling pathway governing PMN transmigration. As shown in Fig. 2, we found that only basolateral colonization of T84 monolayers with *S. flexneri* elicited a marked neutrophil trans epithelial migration (i.e., PMN which had completely traversed the monolayer) response in the basolateral-to-apical direction. In sharp contrast, comparable apical exposure to *S. flexneri* failed to stimulate detectable PMN trans epithelial migration. These data suggest that only *S. flexneri* exposed to the basolateral surface, rather than to the apical surface, of intestinal epithelia initiate the signals required for PMN trans epithelial migration.

Thus, having established that association of *S. flexneri* with the epithelial basolateral membrane domain is an important determinant for eliciting PMN trans epithelial migration, we investigated whether epithelial cell contact with or simply basolateral exposure to soluble *S. flexneri* products was essential for induction of PMN trans epithelial migration. As shown in Fig. 3, exposure of the basolateral membrane to *S. flexneri* products under conditions which prevented direct *Shigella*-epi-

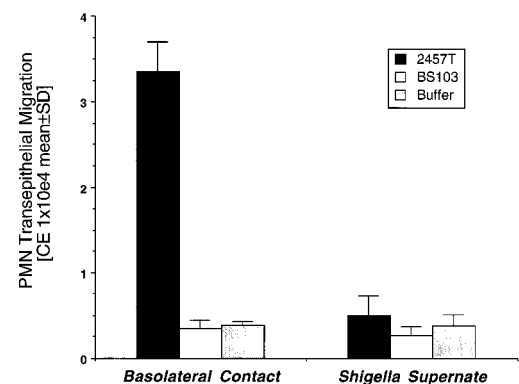


FIG. 3. Effect of *S. flexneri*-intestinal epithelial cell contact on the ability to induce PMN trans epithelial migration. *Shigella*-induced PMN trans epithelial migration is compared to conditions in which epithelia were exposed to *Shigella* soluble products at the basolateral membrane interface but in which no direct *Shigella*-epithelial cell basolateral membrane contact existed. Results are expressed as the mean and SD of triplicate values for each condition and are representative of one of three experiments showing similar results.

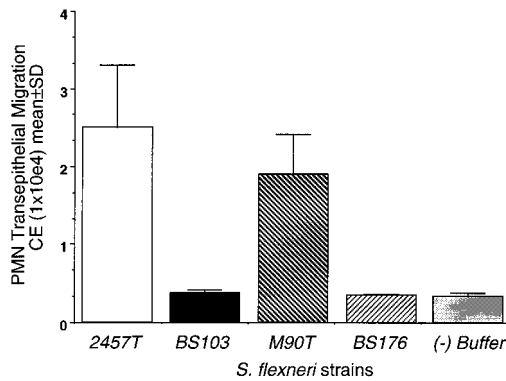


FIG. 4. Comparison of isogenic pairs of plasmid-carrying and plasmid-cured strains of *S. flexneri*. The negative control (-) (gray bar) represents PMN transmigration to HBSS(+) buffer in the absence of bacteria or a chemotactic stimulus. A positive control was established as described in the legend to Fig. 2 ($[30.72 \pm 5.32] \times 10^4$ CE). Data are means and SD for four monolayers in a single experiment and are representative of four separate experiments, all showing the same result.

thelial cell contact was ineffective in inducing PMN transepithelial migration. Thus, in the absence of direct bacterium-basolateral membrane contact, PMN transepithelial migration was reduced about 10-fold.

***S. flexneri*-induced PMN transepithelial migration requires the *Shigella* virulence plasmid.** The virulence of *Shigella* spp. requires expression of genes present on the chromosome, as well as the large virulence plasmid (220 kb), which harbors all of the genes required for invasion (26). We next sought evidence to determine whether the ability to induce the signaling cascade(s) which mediates PMN trafficking across intestinal epithelial cell monolayers is dependent on the presence of the *Shigella* virulence plasmid. Wild-type *S. flexneri* (2457T) was compared to its plasmid-cured avirulent derivative (BS103) for the ability to initiate the transepithelial signals required for directing PMN migration in the basolateral-to-apical direction. As shown in Fig. 2, exposure of *S. flexneri* 2457T to only the basolateral surface specifically induced a directed subepithelial cell-to-lumen PMN transmigration response, while the avirulent strain BS103 failed to elicit PMN transepithelial migration when interfaced with either epithelial membrane domain, despite normal attachment. To address the possibility that such results are strain dependent, we surveyed an additional isogenic pair of *S. flexneri* strains, wild type (M90T) and plasmid cured (BS176), for their ability to induce transepithelial signaling to PMN. Consistent with the results observed above, we found that only the wild-type strain (M90T) was able to specifically elicit the necessary signal pathway to subepithelial PMN whereas the plasmid-cured avirulent strain (BS176) failed to generate transepithelial signaling to PMN (Fig. 4). These data strongly indicate that the ability to induce PMN signals is dependent on genes on the *Shigella* virulence plasmid since the loss of the virulence plasmid correlated with the loss of PMN signaling.

Basolateral access is required for *S. flexneri* to induce PMN transepithelial migration. We next sought to investigate whether pathophysiological events which lead to perturbations of epithelial tight junctions could facilitate the generation of signals required for physiologically directed PMN transepithelial migration. Thus, to permit luminal (apical) *Shigella* access to the basolateral membrane, confluent T84-cell monolayers were transiently perturbed by extracellular Ca^{2+} depletion (38). Such treatment disrupts intercellular tight junctions, resulting in epithelial cell depolarization while preserving the columnar ar-

chitecture of the intestinal epithelial cells and permitting access of lumenally applied bacteria to basolateral surface ligands (38). As shown in Fig. 5, only under conditions where epithelial tight junctions were perturbed did apical epithelial exposure of wild-type *Shigella* induce neutrophil transepithelial migration in the basolateral-to-apical direction. In contrast, noninvasive *Shigella* remained unable to induce PMN transepithelial migration whether or not intercellular tight junctions were perturbed. These data suggest that during events which lead to the disruption of epithelial tight junctions, lumenally restricted *Shigella* is able to gain access to the basolateral epithelial membrane domain required for cell entry and evoke the generation of signals essential for eliciting PMN transepithelial migration.

The ability of *Shigella* to induce signals to PMN requires a functional type III secretion system and/or invasion. The invasive capacity of *Shigella* depends upon proteins encoded by three contiguous operons (*ipa*, *mxi*, and *spa*) in a 31-kb region on the virulence plasmid. The *ipa* locus encodes a set of secreted proteins (Ipa) which are effectors of the entry process (17, 33), while the *mxi* and *spa* loci encode the specialized type III secretion apparatus for export of Ipa proteins (1–4, 54). Having established that the *Shigella* virulence plasmid is necessary to promote signaling to PMN, we investigated whether *Shigella* invasion and/or the functional type III secretion apparatus is a key component in generating PMN transepithelial signal cascades. Thus, to examine the mechanisms by which *S. flexneri* induces PMN transepithelial migration, we examined the ability of *S. flexneri* mutants to induce signals to subepithelial PMN. All five mutants tested were unable to synthesize different proteins required for invasion into epithelial cells. As shown in Fig. 6, basolateral exposure of strains BS232, BS545, and BS547, which harbor independent mutations in the type III secretion apparatus (Table 1), failed to elicit PMN transepithelial migration. BS228, a noninvasive mutant that is secretion competent but synthesizes only a truncated form of IpaB and no IpaD, IpaC, or IpaA, does not elicit PMN transepithelial signaling. Likewise, 2457O, a *virF* mutant which does not make Ipa proteins or the Mxi-Spa secretion apparatus, failed to generate transepithelial signals required for directed PMN transepithelial migration. It is important to note that although *S. flexneri* BS228 is not able to invade epithelial cells, it adheres to epithelial cells to a greater extent than 2457T does (data not shown), yet BS228 failed to induce PMN transepi-

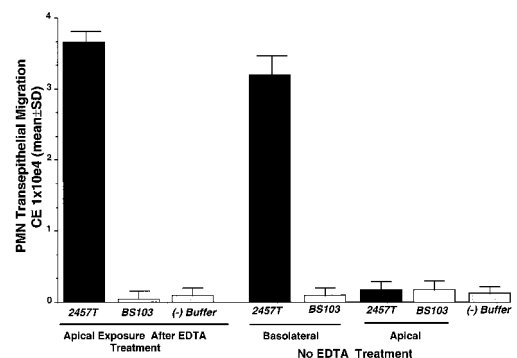


FIG. 5. Effects of EDTA treatment on the ability of apically associated *S. flexneri* to induce PMN transepithelial migration. Diffuse disruption of tight junctions was obtained by means of a brief EDTA exposure. The negative control (-) represents PMN transmigration to HBSS(+) buffer in the absence of bacteria or a chemotactic stimulus. A positive control was established as described in the legend to Fig. 2 ($[33.34 \pm 4.21] \times 10^4$ CE). Data are means and SD for four monolayers in a single experiment and are representative of four separate experiments, all showing the same result.

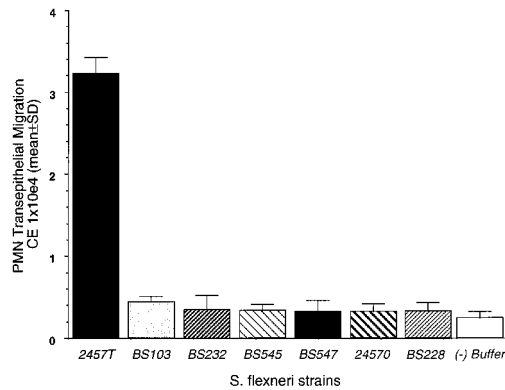


FIG. 6. The ability of *S. flexneri* to induce PMN transepithelial migration is dependent upon a functional type III secretion apparatus. The negative control (–) (open bar) represents PMN transmigration to HBSS(+) buffer in the absence of bacteria or a chemotactic stimulus. A positive control was established as described in the legend to Fig. 2 ($[15.21 \pm 2.72] \times 10^4$ CE). Data are means and SD for four monolayers in a single experiment and are representative of four separate experiments, all showing the same result.

thelial migration. These data suggest that failure of BS228 and other mutant strains of *Shigella* to induce PMN transepithelial migration is not due to the inability of these strains to attach to the epithelial basolateral surface. Therefore, while basolateral exposure of wild-type strain 2457T induced a directed basolateral-to-apical PMN transmigration response, exposure to any one of the invasion-defective strains, irrespective of the mutation, failed to elicit PMN transepithelial migration. These data are consistent with our earlier observation that avirulent, plasmid-cured *S. flexneri* strains do not initiate the signals important for inducing the PMN transepithelial migration response.

DISCUSSION

Shigella species are the causative agents of bacillary dysentery, a disease characterized by bacterial invasion of and multiplication within human colonic epithelial cells (5, 45). *Shigella* binding to epithelial cells engages a complex signaling cascade which includes bacterium-mediated endocytosis, lysis of the membrane-bound phagocytic vacuole, growth of the bacteria within the cytoplasmic compartment, and intercellular spreading by using the host cytoskeleton as a motor (6, 13, 25, 45). Moreover, recent studies have indicated that in both in vitro (35) and in vivo (41) models of *Shigella* invasion of intestinal epithelium, invasion occurs preferentially from the basolateral epithelial membrane domain. Hence, the ability of invasive bacteria to reach the basolateral surface of enterocytes seems to be a crucial step in *Shigella* pathogenesis and is consistent with the observation that invading *Shigella* bacteria which reach the underlying lamina propria evoke an intense inflammatory response (11). Therefore, we sought to determine whether interfacing *Shigella* with the basolateral epithelial membrane domain of polarized intestinal T84 epithelial cells (i.e., the same surface mediating *Shigella* invasion) promoted the generation of discrete signals necessary to elicit PMN transepithelial migration. These findings identify three integral components of *Shigella* pathogenesis essential for the initiation of signals required for the movement of PMN across cultured intestinal epithelial monolayers. First, this signaling response to subepithelial PMN exhibited a strict dependence on *Shigella* contact with the epithelial basolateral membrane domain. Second, such signaling required the participation of the 220-kb virulence plasmid. Third, the ability of *Shigella* to induce signals involved

in the transepithelial migration of PMN requires a functional type III secretion apparatus in which Ipa proteins are essential.

Little is known about the nature of *Shigella*-induced signaling cascades and protein(s) directly involved in evoking the transepithelial signal to neutrophils during active states of enterocolitis. A relevant paradigm is the concept that epithelial orchestration of PMN movement induced by *Salmonella typhimurium* is mediated by polarized secretion of distinct chemokines (28–30). After apical epithelial cell-*S. typhimurium* contact, the intestinal epithelium secretes chemokines which play an essential role in recruiting PMN from the peripheral circulation and directing them to migrate across the epithelium to the intestinal lumen (28–30). Unlike *S. typhimurium*, the capacity of *S. flexneri* to reach the basolateral surface of enterocytes is an essential step in *Shigella* pathogenesis. How *S. flexneri* penetrates the intestinal mucosa to reach the basolateral surface of the epithelium has been an area of intense interest. One possibility is that *Shigella* bacteria interact with specialized cells, termed M cells, which lie over the Peyer patches (41, 46, 55). These cells function to continually endocytose macromolecules as well as microorganisms from the intestinal lumen. Thus, as a result of M-cell-mediated entry, *Shigella* bacteria are able to gain access to the basolateral epithelial cell domain. Consistent with these observations, our studies demonstrate that only wild-type *S. flexneri* strains, when interfaced at the basolateral epithelial membrane domain of intestinal epithelial cell monolayers, generated the appropriate set of signals to drive PMN across the intestinal epithelium. Given that the critical step in *Shigella* pathogenesis is the ability of the organism to access the basolateral surface of enterocytes, it is not surprising that we were unable to detect measurable amounts of PMN transmigration on exposure of *Shigella* bacteria to the apical epithelial membrane domain. Our results, however, are in contrast to the findings of Perdomo et al. (42), who reported that apical surface contact by wild-type and plasmid-cured strains of *S. flexneri* showed an equal ability to induce PMN to transmigrate through a confluent epithelial cell monolayer.

Since only virulent *S. flexneri* could induce PMN transepithelial migration, we were able to examine *Shigella*-elicited factors which might drive this response. Notably, we found that *Shigella*-induced PMN transmigration is dependent upon the presence of the 220-kb virulence plasmid. Strains cured of this plasmid are avirulent and noninvasive and, as we demonstrate in this study, fail to induce signaling to subepithelial PMN. Since Perdomo et al. (42) could not detect this difference in behavior between virulent and avirulent *S. flexneri* strains, our data reveal a previously unrecognized phenotype associated with *Shigella* virulence (i.e., the ability to induce PMN transepithelial migration). Such conflicting results cannot be attributed to strain differences. We used the same isogenic pair of plasmid-containing and plasmid-cured strains (M90T and BS176, respectively) as Perdomo et al. (42) and found no PMN induction with the plasmid-cured strain. However, there are two principal differences between our investigation and that by Perdomo et al. First our studies assessed *Shigella*-epithelial cell interactions from the basolateral membrane domain rather than the apical membrane domain. Given that the entry of *S. flexneri* into the intestinal barrier occurs via the M cells of follicle-associated epithelium and given that the ability of invasive *Shigella* to reach the basolateral surface of enterocytes seems to be a crucial step in *Shigella* pathogenesis, we reasoned, and show in this report, that *Shigella*-basolateral epithelial cell interactions have a profound effect on the ability of the bacteria to induce an inflammatory response. Second, the interpretation of PMN transepithelial migration differs between our

study and that of Perdomo et al. (42). In the gastrointestinal tract, active inflammatory disease characterized by migration of neutrophils across the epithelial lining is a hallmark of both chronic and self-limited diseases (20, 58). To reach the epithelial surface, PMN must travel out of the vascular blood where they encounter matrix components, basement membrane, and finally the epithelium. Still, to cross the intestinal epithelium, PMN must traverse the paracellular space, impale epithelial tight junctions, and move into the luminal compartment (20, 37, 43, 51, 58), where they can interact with the apical epithelial membrane. The sum of these events results in crypt abscesses. In patients with active inflammation, the degree of PMN transepithelial migration correlates with the severity of symptoms (20, 58). Therefore, we define PMN transepithelial migration according to this histopathological definition as those PMN which have completely traversed the monolayer, impaled epithelial tight junctions, and are thus located in the apical epithelial cell compartment. In contrast, Perdomo et al. measured PMN which were associated only with the monolayer (filter) at the basolateral pole, since the number of PMN that traversed the monolayer and appeared in the apical reservoir was too small to be detected and thus largely represents PMN which have migrated only into the monolayer but have not yet crossed the tight junction and hence are trapped in the paracellular and subepithelial spaces (20, 39, 40, 58).

Genes in the *Shigella ipa* operon (*ipaBCD*) play crucial roles in the invasion of epithelial cells by *Shigella*. Mutants unable to synthesize the Ipa proteins not only are incapable of eliciting rearrangement of the actin cytoskeleton around bacterial attachment sites on epithelial cells but also are incapable of disrupting the phagocytic vacuoles surrounding invading bacteria. Secretion of Ipa invasins into the bacterial environment is mediated by the Mxi and Spa proteins (1–4, 54), which form a type III protein secretion system (53). Moreover, secretion of Ipa invasins from *Shigella* occurs more efficiently upon contact with the basolateral surface of polarized intestinal epithelial cells (32, 57). Although plasmid-cured strains of *Shigella* failed to elicit transepithelial signaling to PMN, it was not clear whether this signaling event was dependent on the genes in the contiguous *ipa*, *mxi*, and *spa* operons. We show that *Shigella* strains which either harbor independent mutations in the type III secretion apparatus or fail to make Ipa proteins were unable to initiate the signals required for directed PMN transepithelial migration. Collectively, these observations strongly suggest that transepithelial signaling to PMN is a central virulence mechanism for *Shigella*-elicited enteritis and depends on plasmid-located genes involved in tissue invasion and secretion of bacterial proteins that mediate invasion. Future experiments should resolve the respective contributions of invasion and type III secretion dependence, imperative for the induction of signals involved in PMN transepithelial migration.

This work identifies a novel *Shigella* virulence mechanism. We demonstrate that upon *S. flexneri*-basolateral epithelial cell interactions, basolateral-to-apical directed PMN transepithelial migration ensues, reflecting events relevant to those which occur in the human intestine during acute stages of inflammation. We have previously developed an in vitro model of PMN-intestinal epithelium interactions to investigate the influence of contact of *S. typhimurium* with intestinal epithelial apical membranes on the subsequent inflammatory response. While we do not completely understand how *Salmonella* and *Shigella* induce inflammatory responses, if differences exist they may be because the host cellular events and the signals elicited upon infection by these microorganisms are distinct. For example, *Shigella* entry into epithelial cells occurs from the basolateral pole and appears to be mediated by the small GTPase rho (56)

while *Salmonella* entry occurs predominantly from the apical pole and is mediated by another GTPase, CDC42 (7). What is clear, however, is that interactions between intestinal epithelial cells and enteric pathogens play a key role in orchestrating the inflammatory response. Significantly, future studies will add to our understanding of the molecular mechanisms important in the regulation of active inflammation characterized by shigellosis and salmonellosis. Perhaps by studying *Shigella* we will add to our understanding of *Salmonella* inflammation and vice versa. Furthermore, the most interesting studies may be those that show differences between these important bacterial pathogens.

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