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Attachment of a Noninvasive Enteric Pathogen, Enteropathogenic *Escherichia coli*, to Cultured Human Intestinal Epithelial Monolayers Induces Transmigration of Neutrophils

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An intense inflammatory cell infiltrate, consisting primarily of polymorphonuclear leukocytes (PMN), accompanies enteric infection by enteropathogenic *Escherichia coli* (EPEC). The mechanism(s) by which this pathogen elicits PMN recruitment has not been studied. To determine whether EPEC infection of intestinal epithelial cells induces PMN to transmigrate, an in vitro model consisting of cultured human intestinal epithelial monolayers (T_{84}), a human EPEC strain (E2348/69), and isolated human PMN was used. Results of these studies showed that EPEC attachment to T_{84} monolayers stimulated the transepithelial migration of PMN in a dose-dependent fashion. This event was not attributable to the classic bacterial chemoattractants, n-formylated peptides, or other soluble bacterial factors. A nonadherent EPEC strain, JPN15, was unable to cause PMN to cross the epithelial monolayer. Epithelial protein synthesis was required for maximum EPEC-induced PMN transmigration to occur. Transfer assays demonstrated the presence of a chemokine in sterilized medium from infected monolayers. Neutralizing antibodies to interleukin 8 ablated ~50% of the chemotactic activity. Studies with EPEC mutant strains revealed that the *eaeB* gene, required for the activation of signal transduction pathways in host cells, was crucial for eliciting PMN transmigration. These data show for the first time that attachment of a noninvasive enteric pathogen to intestinal epithelial cells induces PMN transmigration. These findings strongly suggest that EPEC attachment to target host cells activates a signal transduction cascade which ultimately leads to the expression and release of an epithelium-derived chemotactic factor(s) for PMN.

The diarrhea associated with infection of the intestinal epithelium by enteropathogenic *Escherichia coli* (EPEC) is an intriguing phenomenon. Although EPEC was recognized as an enteric pathogen half a century ago, the mechanisms involved in production of disease remain undefined. Unlike other pathogenic *E. coli* bacteria which claim toxin production or invasion as virulence factors, EPEC possesses neither of these (28). EPEC, in contrast, appears to be quite savvy in its approach to pathogenesis. By complex interactions between plasmid and chromosomal genes, EPEC is capable of inducing significant biochemical and morphological changes in host target cells. Biochemical alterations include a dramatic elevation in intracellular calcium concentration (3, 10) and activation of a number of protein kinases eventuating in the phosphorylation of several host cell proteins (2, 32, 42). We have previously shown that these EPEC-induced alterations in host cell signaling correlate with significant changes in intestinal epithelial function (45).

EPEC attachment to host intestinal epithelial cells is key for pathogenesis. Although EPEC attachment to host cells induces degeneration of microvilli, loss of brush border enzymes appears not to be responsible for the associated diarrhea (11, 12, 18). Attachment occurs in a stepwise fashion initially requiring the expression of the bundle-forming pilus (bfp) which is encoded on the 60-Mb pMAR2 plasmid (17). Attachment conferred by the bfp is termed nonintimate. Intimate attachment and formation of the attaching/effacing lesion, which is characteristic of EPEC infection, require the expression of a 94-kDa outer membrane protein called intimin (22, 23). Intimin is encoded by a chromosomal gene called *eaeA*. A second chromosomal gene, *eaeB*, is located downstream from the *eaeA* gene and encodes a 39-kDa soluble protein. This product has now been shown to be necessary for both intimate attachment and signal transduction activity (9, 14).

The morphological lesion associated with EPEC infection is characterized by a "cup-and-pedestal" formation of the epithelial cell membrane at the site of attachment (26). Underlying this lesion is an aggregate of host cell cytoskeletal proteins consisting of actin, myosin, and others (13, 27). Examination of gross histological specimens from the intestines of infected animals reveals additional findings. Notably, extensive infiltration by inflammatory cells, the majority being polymorphonuclear leukocytes (PMN), is seen. PMN are not only found in the lamina propria but also have crossed intact epithelial crypts and accumulated in the intestinal lumen (37, 47, 48). In view of the substantial inflammatory cell infiltrate associated with EPEC infection, it is likely that PMN play a role in the production of diarrhea.

The transmigration of PMN across an intact epithelial barrier is not a random occurrence. Instead, there are specific chemoattractive factors that are responsible for directing the movement of PMN out of the vasculature, into the lamina propria, and finally across the epithelium. PMN chemotaxis may occur in response to specific bacterial factors, the classic one being the n-formylated peptide formyl-methionyl-leucyl phenylalanine (fMLP) (16). Alternatively, bacterial infection may induce the expression of epithelium-derived factors such

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TABLE 1. Correlation of specific EPEC virulence genes and epithelial cell function

Strain	Genetic characteristics[a]			PMN transmigration (no. of PMN [10^4])[b]	Barrier function (% change in resistance)[b]
	bfp	*eaeA*	*eaeB*		
WT[c]	+	+	+	12.8 ± 1.4	−50 ± 4
JPN15	−	+	+	1.6 ± 0.3	10 ± 5
CVD206	+	−	+	13.7 ± 4.0	−25 ± 7
UMD864	+	+	−	2.5 ± 0.4	13 ± 10

[a] bfp, bundle-forming pilus encoded on the pMAR2 plasmid; *eaeA*, gene encoding intimin; *eaeB*, gene encoding 39-kDa protein. +, present; −, absent.
[b] Values are means ± SEMs.
[c] Wild-type EPEC E2348/69.

as interleukin-8 (IL-8), leukotriene B_4, and platelet activating factor (41). Several bacterial pathogens have now been shown to stimulate the expression of IL-8 (4, 24, 33), which may in part be responsible for the associated inflammatory cell infiltrate. Whether EPEC attachment to intestinal epithelial cells induces PMN transmigration has not been investigated.

Here, we examine the impact of attachment of a noninvasive enteric pathogen, EPEC, to human intestinal epithelial cells on PMN transmigration by using an in vitro model system previously described by others (40). These studies show that EPEC bacteria induce PMN to transmigrate in the physiological (basolateral-to-apical) direction. Stimulation of this process is not via classical n-formylated peptide signaling, which is often responsible for attracting PMN to bacteria. Instead, adherence of EPEC to the host target cell and subsequent activation of epithelial signaling pathways are required.

MATERIALS AND METHODS

Cell culture. T_{84} cells were a generous gift from Kim Barrett, University of California, San Diego. Passages 37 to 52 were used for these studies and were grown in a 1:1 (vol/vol) mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 as described previously (31).

Construction of inverted monolayers. Inverted monolayers were constructed as originally described by Parkos et al. (40). In brief, 1-mm-high polycarbonate rings with a diameter equal to that of the base of 0.33-cm^2 Transwell filters (Costar, Cambridge, Mass.) were glued to the bottom with RTV silicone rubber glue (General Electric Co., Wilmington, Mass.). This product is nontoxic after setting and is nonconductive. Filters with a 5-μm pore size were crucial for allowing permeation by PMN. Inverted filters were coated with collagen, and T_{84} cells were plated and allowed to attach overnight. The inserts were then flipped and placed into 24-well culture plates. After 6 to 14 days, monolayers developed stable electrical resistances and were used for experiments.

Bacterial strains. The EPEC strains used in these studies are summarized in Table 1. E2348/69 is a wild-type strain of EPEC which demonstrates localized adherence to HEp-2 cells (39). JPN15 is an isogenic strain that has spontaneously lost the plasmid pMAR2 (17). Previous work from our lab has shown that JPN15 is essentially nonadherent to T_{84} cells and, as a result, has no effect on electrical resistance (45). CVD206 is a derivative of the wild-type strain E2348/69 in which 1,847 bp (~66%) of the *eaeA* gene, located within the chromosomal pathogenic locus (35), have been deleted (6). As a result, this mutant is incapable of producing intimin (22) yet still demonstrates nonintimate adherence (23) and some signal-transducing activity (15, 42). E2348/69, JPN15, and CVD206 were generous gifts from James Kaper, Center for Vaccine Development, University of Maryland, Baltimore. Strain UMD864 is an *eaeB* deletion mutant (9). This gene encodes a 39-kDa soluble protein which is important for both intimate attachment and activating signal transduction in epithelial cells (9, 14). Strain UMD864 was kindly provided by Michael Donnenberg, Infectious Diseases, University of Maryland, Baltimore.

Bacterial attachment to inverted T_{84} intestinal epithelial monolayers. Inverted monolayers were lifted from wells, drained of medium, and gently washed with Hanks balanced salt solution (HBSS) containing Ca^{2+}, Mg^{2+}, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4 [HBSS(+)]; GIBCO-BRL). The monolayers were placed into new 24-well plates with 1.0 ml of HBSS(+) in the basolateral reservoir and 0.05 ml in the apical reservoir. Overnight cultures of EPEC in Luria-Bertani (LB) broth were diluted (1:33) in serum- and antibiotic-free T_{84} medium containing 0.5% mannose and grown to the mid-log growth phase (optical density at 660 nm, ~0.8; 5×10^8 bacteria per ml). The bacterial suspension was centrifuged at 14,000 rpm for 3 min and washed three times in HBSS without Ca^{2+} and Mg^{2+}. Bacteria were resuspended in HBSS(+) containing 0.5% mannose and layered onto the cells at a concentration of $\sim 2 \times 10^8$ per monolayer unless stated otherwise. Bacterial attachment was allowed to proceed for 60 min in a humidified chamber at 37°C. Unattached bacteria were removed by washing five times in HBSS(+). The number of attached EPEC bacteria were determined by exposing the monolayers to 0.1 ml of 1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.), diluting the samples, and plating on LB agar plates. The CFU were counted, and the numbers of attached bacteria per monolayer were calculated.

Isolation of human neutrophils. Neutrophils were isolated from the blood of healthy volunteers. Citrate-anticoagulated blood was diluted 1:5 in Ca^{2+}- and Mg^{2+}-free HBSS, layered on top of Histopaque 1077 (Sigma), and centrifuged at $400 \times g$ for 30 min at 20°C. The pellet containing erythrocytes and neutrophils was diluted in HBSS without Ca^{2+} and Mg^{2+}. Erythrocytes were pooled by adding 5% dextran for 30 min at 4°C and then lysed by the addition of 0.155 M NH_4Cl. Neutrophils were washed and resuspended in HBSS(+) to achieve a density of 2×10^7 cells per ml.

EPEC-induced PMN transepithelial migration. The physiologically directed (basolateral-to-apical) PMN transepithelial migration assay has been detailed previously by others (29, 40). Before infection with EPEC, T_{84} monolayers were washed and kept for 3 h in serum- and antibiotic-free T_{84} medium. The infected monolayers were then transferred, apical side down, into a 24-well tissue culture tray with 1.0 ml of HBSS(+) containing 0.1% glucose. Isolated PMN (10^6 in 50 μl) plus 150 μl of HBSS(+) were added to the basolateral side (upper reservoir) of each monolayer and incubated for 110 min at 37°C. Positive controls for each transmigration assay were represented by the transmigration response to 1 μM fMLP.

Chemotaxis assay. Transmigration of neutrophils was assessed by quantitation of myeloperoxidase as described previously by Parkos et al. (40). Briefly, at the end of the experiment, the T_{84} monolayers were cooled to 4°C, washed with HBSS(+), and solubilized in HBSS containing 0.5% Triton X-100 to determine the number of monolayer-associated PMN. The pH was adjusted to 4.2 with a 1:10 dilution of 1.0 M Na citrate. Peroxidase activity was assayed by the addition of an equal volume of 1 mM 2,2′-azino-di-(3-ethyl) dithiazoline sulfonic acid and 10 mM H_2O_2 in 100 mM citrate (pH 4.2). To quantitate the number of neutrophils that had completely transmigrated the epithelial monolayer and were present in the reservoir, Triton X-100 was added directly to the reservoir and myeloperoxidase activity was measured as described above. The number of attached EPEC bacteria was determined from duplicate monolayers. The subsequent color reactions were quantitated spectrophotometrically at 405 nm. PMN cell equivalents were calculated from a standard curve established by using known numbers of PMN. Standard curves were constructed for each experiment. T_{84} monolayers were found to have no significant myeloperoxidase activity in the absence of neutrophils.

Gentamicin and chloramphenicol treatment of EPEC. After the 1-h colonization step described above, EPEC-infected monolayers were exposed to gentamicin (50 μg/ml) for 20 or 40 min to eliminate the adherent bacteria. Monolayers were then washed five times in HBSS(+) to remove residual gentamicin, returned to the 24-well tray, and subsequently assayed for PMN transmigration as detailed above. To determine whether EPEC had invaded T_{84} cells, duplicate EPEC-infected T_{84} monolayers were treated as described above with gentamicin and then lysed with 1% Triton X-100 for 20 min. Cell lysates were cultured on agar plates, and colonies were counted. Such treatment does not affect bacterial viability (5). In other experiments, EPEC bacteria were pretreated with chloramphenicol (100 μg/ml for 45 min), a bacteriostatic antibiotic which inhibits protein synthesis of prokaryotic but not eukaryotic cells, before addition to T_{84} monolayers. EPEC bacteria were exposed to chloramphenicol throughout the chemotaxis assay.

Cycloheximide treatment of T_{84} monolayers. Cycloheximide (Sigma) was used to inhibit eukaryotic protein synthesis without affecting prokaryotic protein synthesis. Before incubation with EPEC, T_{84} monolayers were treated for 3 h with 2 μg of cycloheximide per ml. This concentration has been found to inhibit 75% of radiolabeled leucine incorporation into T_{84} cell-precipitable protein (36). The effect of cycloheximide on EPEC-induced PMN transmigration was then determined.

tBOC-fMLP pretreatment of PMN. Isolated PMN (2×10^7/ml) were preincubated with the n-formyl peptide receptor antagonist *N-tert*-butoxycarbonyl-1-methionyl-1-leucyl-1-phenylalanine (tBOC-fMLP; Sigma) at a concentration of 300 μM for 5 min at 37°C. tBOC-fMLP-pretreated PMN were then utilized for PMN transmigration experiments and assayed as described above.

Preparation of EPEC culture supernatant (EPEC buffer). Overnight cultures of EPEC were diluted (1:33) in serum-free medium containing 0.5% mannose and grown to an optical density at 600 nm of ~0.8. The medium was spun free of bacteria (14,000 rpm for 3 min), and the supernatant (termed EPEC buffer) was collected and passed through a 0.2-μm-pore-size filter before use in select experiments.

Transfer assays. T_{84} monolayers on Transwell filters were infected with EPEC for 3 h as described above. The medium was then collected and sterilized by using a 0.2-μm-pore-size filter. To determine the chemotactic activity of this medium, its ability to induce transmigration of isolated PMN across bare Transwell filters was tested. Medium collected from infected monolayers was placed in the lower

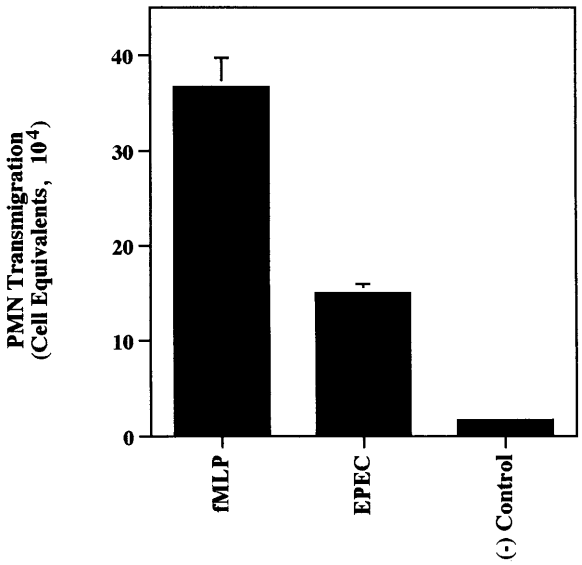

FIG. 1. Transepithelial (basolateral-to-apical) migration of PMN across T_{84} monolayers in response to apical colonization by EPEC. The potent chemotactic peptide fMLP was used as a positive control for these experiments. The negative control consisted of basolaterally positioned PMN in the absence of a chemoattractive gradient. In contrast to the negative control, apical colonization of T_{84} cells by EPEC elicited a significant transmigration response. Data represent the means ± SEMs (n = 4 to 6; P = 0.0002 for EPEC versus negative control).

reservoirs of tissue culture wells containing the Transwell inserts, and isolated PMN (10^6) were placed in the upper chamber. The number of PMN which had transmigrated after 1 h was then quantitated by the myeloperoxidase assay detailed above. The effect of neutralizing antibody to IL-8 (30 μg/ml; Genzyme, Cambridge, Mass.) on the chemotactic activity of medium from EPEC-infected T_{84} monolayers was determined. Antibody was allowed to incubate with the medium for 20 min prior to the addition of PMN.

Statistical analysis. All data are represented as means ± standard errors of the means (SEMs). Data comparisons were made with Student's t test. Differences were considered significant when P was <0.05.

RESULTS

Infection of T_{84} monolayers with EPEC induces PMN to transmigrate. Because a significant infiltration of PMN occurs in vivo in response to EPEC infection, we investigated whether this phenomenon could be modeled in vitro. By using an inverted monolayer system previously described by others (40), the apical aspect of the cultured monolayers could be infected with EPEC and isolated human PMN could be added to the physiologically appropriate (basolateral) surface. Attachment of wild-type EPEC to the apical membrane of T_{84} cells induced the basolaterally to apically directed transmigration of PMN (Fig. 1). In fact, PMN transmigration induced by EPEC was 42% as effective as that which occurred in response to the potent chemoattractant fMLP [(36.7 ± 3) and (15 ± 1) × 10^4 PMN cell equivalents per monolayer for fMLP and EPEC, respectively]. This number represents PMN that have completely transmigrated the monolayers and are present in the opposite reservoir from which they were added as well as PMN that have infiltrated the epithelial monolayer but not yet impaled the tight junctions, thus remaining associated with the monolayer. In general, the number of PMN that remain membrane-associated at the end of each experiment is quite low [(1.6 ± 0.6) × 10^4 PMN cell equivalents per monolayer). T_{84} medium from uninfected monolayers (negative control) possessed negligible chemotactic potential.

Dose-response relationship of PMN transmigration to number of attached EPEC. Previous studies from our laboratory utilized a model in which T_{84} monolayers were inoculated with 100 EPEC organisms per epithelial cell. The number of T_{84} cells per monolayer was determined by suspending cells following exposure to trypsin and counting with a hemocytometer. To further characterize this model, the relationship between the concentration of the initial bacterial inoculum and the number of adherent EPEC following a 1-h incubation was determined. After this time, approximately 10 to 20% of the applied EPEC bacteria had attached to the T_{84} cells. As shown in Fig. 2A, incubation of 10, 100, or 1,000 EPEC organisms per T_{84} cell resulted in the attachment of 2, 20, and 100 EPEC organisms per T_{84} cell, respectively. It should be realized that although expressed as number of bacteria per T_{84} cell, this is only an average since EPEC bacteria attach focally as microcolonies of many organisms. In actuality, therefore, only a portion of cells harbor attached organisms.

To determine whether the number of PMN that transmigrate a monolayer is dependent upon the number of attached bacteria, transmigration assays were performed with T_{84} monolayers with 2, 20, or 100 attached organisms per T_{84} cell. As shown in Fig. 2B, the degree of PMN transmigration is closely linked to the number of attached pathogens. For all subsequent experiments, 100 attached EPEC organisms per T_{84} cell was used.

Although invasion is generally not viewed as a pathogenic factor for EPEC, it has been demonstrated to be invasive in some cell culture systems (43). To ensure that PMN transmigration in response to EPEC infection was not caused by intracellular organisms, invasion assays using gentamicin were performed as described in Materials and Methods. By use of this standard assay, essentially no intracellular organisms (0.0001% of the original inoculum) were recovered (assay performed in triplicate in two separate experiments). In addition, careful examination of numerous electron micrographs of EPEC-infected T_{84} monolayers revealed no intracellular organisms. These findings suggest that EPEC does not invade T_{84} cells.

Bacterial-derived n-formyl peptides are not responsible for EPEC-induced PMN transmigration. The n-formylated peptides released from bacteria are well-known chemoattractants of PMN. To determine whether such peptides might be responsible for EPEC-stimulated PMN transepithelial migration, PMN were exposed to the n-formyl peptide receptor antagonist tBOC-fMLP. PMN transmigration elicited by fMLP was significantly inhibited by pretreatment with 300 μM tBOC-fMLP (88.7 and 88.2% for 10 and 100 nM fMLP, respectively) (Fig. 3). In contrast, EPEC-induced PMN transmigration was completely unaltered by this n-formylated peptide receptor antagonist (Fig. 3).

To test whether other released bacterial factors were responsible for EPEC-induced PMN transmigration, the chemotactic potential of sterilized bacterial culture medium (EPEC buffer) was tested. There was no significant difference in the degree of transmigration seen with EPEC buffer and negative controls [(1.05 ± 0.8) and (0.8 ± 0.7) × 10^4 PMN cell equivalents per monolayer for EPEC buffer and negative control, respectively; n = 6, P = 0.8]. These findings suggest that EPEC-stimulated PMN transmigration is not in response to bacterial factors released into the culture medium.

Interactive events required for EPEC-associated PMN transmigration. Since neither tissue culture medium from uninfected T_{84} cells (negative control) nor sterilized medium from bacterial cultures stimulated PMN transmigration, the conclusion that direct interactions between the pathogenic or-

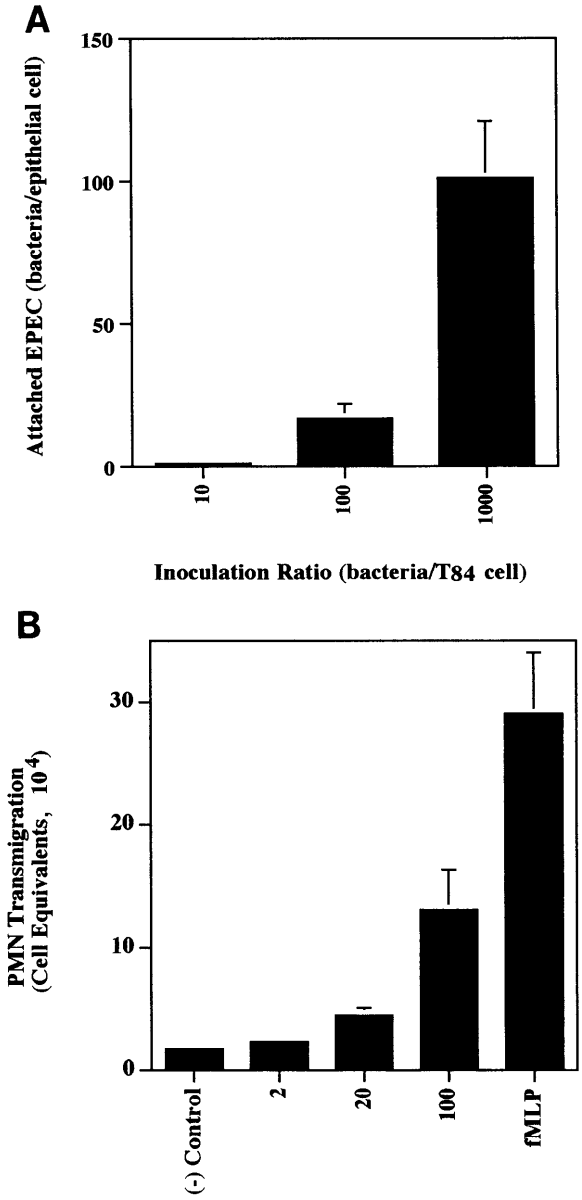

FIG. 2. (A) Dose dependence of inoculum concentration on EPEC attachment to T_{84} monolayers. The number of EPEC organisms which attached to T_{84} monolayers after 1 h increased with the concentration of the original inoculum (inoculation ratio of 10, 100, or 1,000 bacteria to T_{84} cell). Data represent means ± SEMs of three separate experiments (n = 5 to 8). (B) Dose response of EPEC-induced PMN transepithelial migration. Intestinal epithelial monolayers with increasing numbers of attached EPEC induced correspondingly greater numbers of PMN to migrate across the epithelial monolayer. Data are expressed as the means ± SEMs (n = 3 to 6).

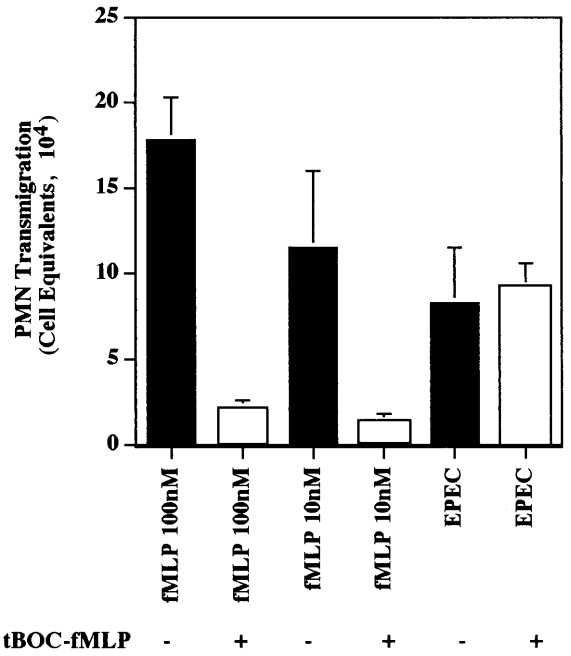

FIG. 3. Effect of PMN n-formyl-peptide receptor antagonist tBOC-fMLP on fMLP- and EPEC-induced transmigration. tBOC-fMLP significantly diminished the PMN transmigration induced by both 100 and 10 nM fMLP but was totally ineffective in preventing EPEC-driven transmigration. Data are expressed as the means ± SEMs (n = 6 to 11). P = 0.00002 for fMLP with or without tBOC-fMLP; P = 0.8 for EPEC with or without tBOC-fMLP.

ganism and intestinal epithelial cells were required was examined in a stepwise fashion. We first questioned whether ongoing bacterial protein synthesis was required to elicit this response. EPEC organisms were exposed to a bacteriostatic antibiotic, chloramphenicol, which inhibits prokaryotic protein synthesis by acting on the 50S ribosomal subunit. Inhibition of bacterial protein synthesis both prior to and during coincubation with T_{84} monolayers had no effect on PMN transmigration (Fig. 4). This finding suggests that active bacterial protein synthesis is not required for EPEC to stimulate PMN transmigration.

To determine the role of eukaryotic protein synthesis in EPEC-stimulated PMN transmigration, T_{84} monolayers were treated with cycloheximide, at 2 μg/ml for 3 h, before the addition of EPEC. This concentration has been demonstrated previously to inhibit 75% of protein synthesis in T_{84} cells (36). Such treatment does not alter T_{84} monolayer resistance as shown by our lab (19). Inhibition of epithelial protein synthesis with cycloheximide resulted in a 42% reduction in PMN transmigration in response to EPEC infection (Fig. 4) yet had no effect on fMLP-induced transmigration. De novo epithelial protein synthesis, therefore, appears to be essential for maximal PMN transmigration to ensue.

To characterize the nature of the interaction between EPEC and the host epithelial cells that ultimately results in the chemotaxis of PMN, we questioned whether the presence of viable EPEC was required or if stimulation of signaling pathways by a brief period of attachment was sufficient to elicit the response. For these experiments, EPEC organisms were allowed to attach to T_{84} monolayers for 1 h and then nonadherent organisms were removed by washing. Attached bacteria were then treated with gentamicin (50 μg/ml) for 20 or 40 min, a time adequate to kill the remaining organisms as determined by plating on LB agar (data not shown), before PMN were added. As shown in Fig. 4, the removal of viable organisms from primed monolayers had no significant impact on EPEC-induced transmigration. These data suggest that a brief period of contact between this enteric pathogen and the epithelial cell is sufficient to trigger the intracellular events that ultimately result in PMN chemotaxis.

Chemotactic activity of medium from EPEC-infected monolayers. To confirm the production and release of a chemotactic factor(s) from EPEC-infected monolayers, transfer assays were performed (Materials and Methods). The sterilized medium from both the apical and basolateral reservoirs of in-

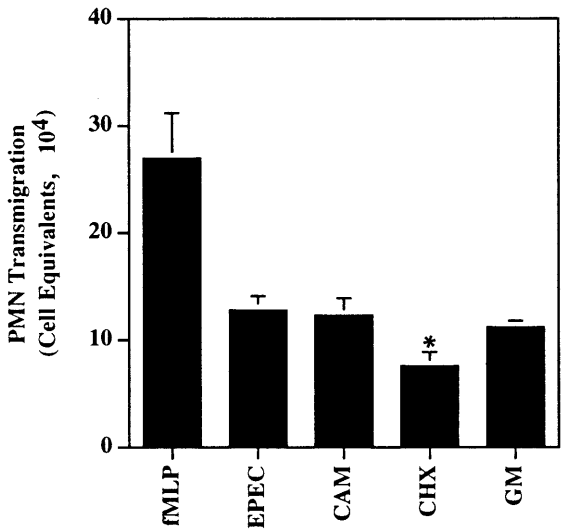

FIG. 4. Effect of prokaryotic and eukaryotic protein synthesis inhibition on EPEC-induced PMN migration. Inhibition of EPEC protein synthesis with chloramphenicol (CAM) had no effect on PMN transmigration ($P = 0.08$). In contrast, inhibition of eukaryotic protein synthesis with cycloheximide (CHX) caused a 42% decrease in transmigration (*, $P = 0.03$) compared with that of EPEC. The requirement for the continuous presence of viable pathogens was examined by eliminating attached EPEC with gentamicin (GM) before adding PMN. T_{84} monolayers primed by a brief exposure to viable pathogens were as effective at driving PMN transmigration as were those colonized by actively replicating organisms. Data represent means ± SEMs of three experiments (n = 5 to 7; $P = 0.4$).

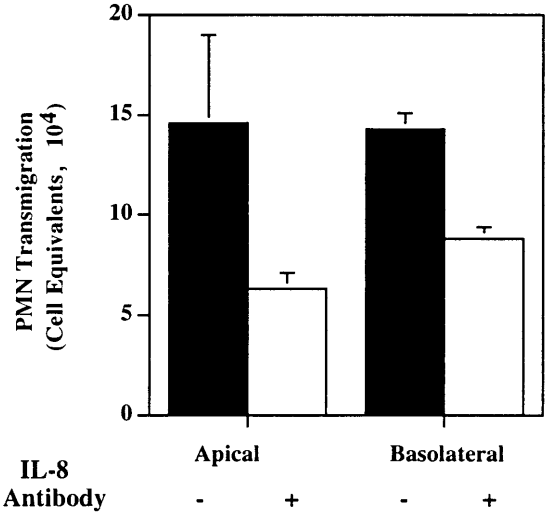

FIG. 5. Medium from EPEC-infected monolayers possesses chemotactic activity. Transfer assays, performed with filters devoid of cells, demonstrated the presence of a chemotactic factor for PMN in sterilized medium collected from both the apical and basolateral reservoirs of infected monolayers. The addition of neutralizing antibody to IL-8 (30 μg/ml) inhibited ~40% of the activity from basolateral medium and ~60% of that from the apical medium. Sterile tissue culture medium, which served as the negative control, induced the transmigration of $(1.9 \pm 1.2) \times 10^4$ PMN.

fected epithelial cells was demonstrated to possess significant chemotactic activity (Fig. 5). Many epithelial cell types have been shown to secrete IL-8, a potent chemoattractant for PMN, in response to bacterial infection. To determine whether IL-8 might be involved in EPEC-associated PMN transmigration, the effect of IL-8 neutralizing antibodies was investigated. Antibodies to IL-8 (30 μg/ml) inhibited PMN transmigration by 43% when added to medium collected from basolateral reservoirs and by 62% when added to medium from apical reservoirs (Fig. 5). This concentration of antibody is sufficient to completely neutralize an IL-8 gradient of 1 ng/ml (33), a level that is rarely secreted in response to bacterial pathogens (24). These findings suggest that IL-8 plays a role in the EPEC-induced inflammatory response but that additional chemotactic factors are likely involved as well.

Role of specific EPEC virulence genes in activating epithelial signals for PMN transmigration. Significant progress in elucidating the virulence genes of EPEC has been made in the past few years. Experiments to examine which bacterial genetic factors may be involved in triggering the signals responsible for PMN transmigration were performed. For these studies, the ability of the various EPEC mutants, described earlier, to induce PMN transmigration was determined (Table 1).

Strain JPN15, which failed to affect resistance, also failed to stimulate PMN transmigration (45). This lack of response is most likely attributable to its inability to attach. Interestingly, despite its attenuated effect on transepithelial resistance (Table 1), the *eaeA* mutant CVD206 was able to induce PMN transmigration to a degree indistinguishable from that seen with the wild-type strain. Regardless of its similarities to CVD206 with regards to attachment, the *eaeB* mutant UMD864 was devoid of chemotactic potential. The inability of the *eaeB* deletion mutant UMD864 to elicit PMN transmigration could not be attributed to differences in attachment. EPEC strains CVD206 and UMD864 attach only in the nonintimate fashion (9). Also, when quantitated, the attachment of CVD206 and UMD864 to T_{84} monolayers did not differ (10.3 ± 9.6 versus 9.9 ± 6.4 attached bacteria per cell). It is most likely, therefore, that the signal transducing factor encoded by the *eaeB* gene is essential for PMN transmigration.

DISCUSSION

In this article, we report for the first time that attachment of a noninvasive enteric pathogen, EPEC, to the apical membrane of intestinal epithelial cells induces PMN transmigration. Although EPEC has been demonstrated to be somewhat invasive in previous reports (43), the differences in our model, namely, use of another cell type, degree of confluence, and the fact that the organisms were not centrifuged onto the cells, may, in part, account for the variability. EPEC-induced PMN transmigration cannot be attributed to the classic bacterial chemoattractants n-formylated peptides, since incubation of isolated PMN with an n-formylated peptide receptor antagonist had no impact on EPEC-induced transmigration. This receptor antagonist, on the other hand, inhibited fMLP-driven transmigration. Similarly, PMN transmigration associated with EPEC infection could not be ascribed to other factors produced by EPEC since sterilized medium from EPEC cultures exhibited no chemotactic potential. This statement, however, must be viewed in the context that the *eaeB* gene product, a secreted 39-kDa protein, appears to be essential for this process. A plausible explanation for this apparent discrepancy may merely be that it is a concentration phenomenon. That is, the local concentration of the EaeB protein secreted at the epithelial cell surface by adherent EPEC is likely much higher than that present in culture filtrates. Consistent with this explanation is that the presence of a nonadherent EPEC strain, JPN15, was not sufficient to elicit transmigration. Rather, EPEC attachment to and the activation of signaling pathways within host cells are required to stimulate this process.

The dependence of PMN transmigration on interactive events between EPEC and host intestinal epithelial cells is similar to that previously described for *Salmonella typhimurium* (33). It is interesting that, although *S. typhimurium* is a much more aggressive pathogen than EPEC with invasive capability, data suggest that it is merely the attachment of this organisms to, and not invasion of, host epithelial cells that is important for stimulating PMN transmigration (33). Such findings may explain why a noninvasive enteric pathogen such as EPEC is as effective at driving PMN across an epithelial layer as is the invasive pathogen *S. typhimurium*.

By employing the exact model system to study EPEC-induced PMN transmigration as that utilized to investigate the mechanisms by which *S. typhimurium* stimulates this event, direct comparisons can be made. For both pathogens, de novo epithelial protein synthesis is key. One difference, however, appears to be the requirement by *S. typhimurium* for the continuous presence of viable organisms. In contrast, our studies with EPEC showed that an abbreviated period of bacterial attachment was sufficient to initiate the cascade of events that ultimately results in the transepithelial summoning of PMN. Once this signaling pathway has been activated, the presence of viable organisms is no longer necessary. This is similar to the observation made by Rosenshine et al. (43) that the initiation of attaching/effacing lesion formation requires only a brief (5- to 10-min) encounter between EPEC and the host cell, after which time viable organisms are no longer needed for the lesion to mature. Possibly, occupation of the epithelial receptor(s) for EPEC by the specific ligand(s), viable or not, may be the key factor. Of note, however, is that the signaling pathways for attaching/effacing lesion formation and PMN transmigration appear to be divergent since the mutant strain CVD206, which is incapable of inducing such lesions, elicits maximal PMN transmigration.

The idea that bacterial attachment to eukaryotic cells can trigger the expression of immunomodulating proteins is not new. Initial studies showed that *E. coli* infection of the urinary tract stimulated IL-6 production (20). An extension of this work demonstrated that urinary epithelial cells colonized with *E. coli* secrete IL-8 (1). In fact, the exposure of bladder epithelial cells to *E. coli* attachment proteins alone was sufficient to trigger the secretion of IL-8 (46). There is now evidence supporting the contention that intestinal epithelial cells are also capable of producing proinflammatory cytokines in response to bacterial attachment. As mentioned earlier, it appears that attachment of *S. typhimurium* to T_{84} monolayers, not invasion, induces the release of IL-8 (33). Also, the noninvasive, toxigenic, enteric pathogen enterohemorrhagic *E. coli* O157 has been shown to increase IL-8 secretion 3- to 12-fold above baseline (24), depending on the cell type employed. In addition, infection of gastric epithelial cells with *Helicobacter pylori*, which rarely if ever invades, results in the secretion of IL-8 (4). Although it does appear that invasive enteropathogens induce the release of much greater concentrations of cytokines from epithelial cells than do noninvasive organisms (24), the question is how much IL-8 is necessary to effect a physiological response such as PMN transmigration. Quantitation of IL-8, or other cytokines, released into the surrounding milieu may not be as meaningful pathophysiologically as is the cellular domain from which the chemotaxin is released, the gradient that is formed, and the concentration that infiltrates and binds to the extracellular matrix underlying the host epithelial cells. Indeed, McCormick et al. have elegantly demonstrated this concept by showing that "imprinted matrices" from *Salmonella*-infected T_{84} monolayers possess chemotactic activity that is inhibited by neutralizing IL-8 antibodies (34). They hypothesize that the basolateral release of IL-8 and its binding to extracellular matrix proteins are important in attracting PMN out of the vasculature and into the lamina propria. Other chemotactic factors may then be responsible for driving PMN across the epithelial layer.

That IL-8 plays a role in EPEC-associated PMN transmigration is suggested by experiments showing that neutralizing antibodies to IL-8 block ~50% of chemotactic activity present in sterilized medium collected from EPEC-infected monolayers. Equivalent levels of chemotactic activity appear to be present in the medium from apical and basolateral reservoirs. Whether equivalent amounts are released from the apical and basolateral domains or an initial gradient exists but equilibrates over time because EPEC attenuates barrier function (45) is not known. The concentration of IL-8 antibody used in our studies has been shown to completely neutralize the effect of a 1-ng/ml gradient of IL-8 and to inhibit 50% of the effect of a 10-ng/ml gradient (33). The concentration of IL-8 released in response to various enteric pathogens in several different cell culture models and native colonic epithelial cells is consistently $\leq$1 ng/ml (24). In fact, the amount of IL-8 secreted in response to noninvasive pathogens is significantly less than this. Assuming that IL-8 is most likely released to some extent into both the apical and basolateral domains, the IL-8 gradient must certainly be below 1 ng/ml. Furthermore, an imposed IL-8 gradient of even 50 ng/ml elicits the transmigration of only 6×10^4 PMN (33), which is approximately half that elicited by either *Salmonella* or EPEC infection of T_{84} monolayers. Taken together, these data suggest that IL-8 does not fully account for the PMN chemotaxis associated with EPEC infection but that other chemoattractants for PMN are involved as well.

In any event, these studies, when examined in light of the previous findings with *S. typhimurium*, suggest that epithelial detection of two enteric pathogens, which are quite different with regards to their virulence characteristics, result in the orchestration of inflammatory responses by similar processes. Further definition of the intracellular signal cascades that bridge pathogenic bacterial attachment to epithelial production of cytokines and finally infiltration by inflammatory cells will help to define whether commonalities or differences prevail.

The other important question to be addressed is which bacterial factors are involved in activating the epithelial signals that result in chemokine production and subsequent PMN transmigration. The studies employing specific EPEC mutants begin to examine this issue. Although EPEC bacteria must attach to stimulate transmigration, neither intimate attachment nor formation of the attaching/effacing lesion is required. The demonstration that the *eaeA* deletion mutant CVD206 is as effective at driving PMN transmigration as is the wild-type strain illustrates this fact. The discrepancy in the ability of CVD206 to stimulate maximum transmigration and yet to alter barrier function only partially is intriguing (Table 1). These findings suggest that the signaling pathways involved in these two events are separate. Intimin, which appears to be involved in rearranging the cytoskeleton, may be more important for perturbing intestinal barrier function, while other EPEC virulence factors are crucial for other pathogenic events. The implication that intimin is not involved in the signaling cascade that stimulates PMN transmigration is confirmed by the observation that the *eaeB* deletion mutant UMB864, which expresses normal amounts of intimin, possesses no chemotactic activity. What is likely more important is the ability of the organism to induce signal transduction events in the host cell. The *eaeA* mutant remains capable of activating host cell tyrosine kinase, damaging microvilli (7, 42), altering the host

cytoskeleton (5, 23), and inducing diarrhea, albeit to a more moderate degree (8). The *eaeB* deletion mutant, on the other hand, appears to possess no signal transduction activity (14). This mutant also fails to alter intestinal epithelial barrier function (Table 1). These findings strongly suggest that EPEC attachment and subsequent activation of epithelial signaling pathways by the *eaeB* gene product are crucial to PMN transmigration. That de novo expression of the EaeB protein may not be required is supported by the finding that chloramphenicol does not affect PMN transmigration. Storage of the 39-kDa EaeB protein within the bacterium and release upon attachment to a host cell would be economical for the pathogen in two ways: (i) the protein would not be produced and released unnecessarily and (ii) there would be no lag time between attachment to host cells and release of this virulence factor. Such protein secretion systems have been described for other bacterial pathogens including *Yersinia*, *Shigella*, and *Salmonella* spp. The type III secretion system directs the secretion of virulence proteins. In the case of *Yersinia* spp., exported bacterial proteins (Yops) are secreted directly into the cytoplasm of the host cell upon contact by use of a type III secretion system (44). There is evidence that EPEC possesses a type III system (21) and that EaeB, which may actually integrate into the host cell membrane or gain access to an intracellular compartment (25), is secreted via such a system.

In summary, the diarrhea that occurs as a result of EPEC infection appears to be the end point of multiple complex and interactive events. The direct effects of this pathogen on an intestinal epithelial function(s), such as barrier function, as well as the effects on other cell populations, such as PMN, which in turn influence epithelial function (30, 38), appear to be important. As studies involving the genetics of EPEC continue to be coupled with studies concerning effects on host cell function, the pathophysiology of EPEC-induced diarrhea may ultimately be understood.

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