Salmonella enterica serovar Typhimurium regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage

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Salmonella enterica serovar Typhimurium regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage. Am J Physiol Gastrointest Liver Physiol 293: G178–G187, 2007; doi:10.1152/ajpgi.00535.2006.—The establishment of tight junctions (TJ) between columnar epithelial cells defines the functional barrier, which enteroinvasive pathogens have to overcome. Salmonella enterica serovar Typhimurium (S. typhimurium) directly invades intestinal epithelial cells but it is not well understood how the pathogen is able to overcome the intestinal barrier and gain access to the circulation. Therefore, we sought to determine whether infection with S. typhimurium could regulate the molecular composition of the TJ and, if so, whether these modifications would influence bacterial translocation and polymorphonuclear leukocyte (PMN) movement across model intestinal epithelium. We found that infection of a model intestinal epithelium with S. typhimurium over 2 h resulted in an ~80% loss of transepithelial electrical resistance. Western blot analysis of epithelial cell lysates demonstrated that S. typhimurium regulated the distribution of the TJ complex proteins claudin-1, zonula occludens (ZO)-2, and E-cadherin in Triton X-100-soluble and insoluble fractions. In addition, S. typhimurium was specifically able to dephosphorylate occludin and degrade ZO-1. This TJ alteration in the epithelial monolayer resulted in 10-fold increase in PMN transepithelial migration. Our data demonstrate that infection with S. typhimurium is associated with the rapid targeting of the tight junctional complex and loss of barrier function. This results in enhanced bacterial translocation and initiation of PMN migration across the intestinal barrier. Therefore, the ability to regulate the molecular composition of TJs facilitates the pathogenicity of S. typhimurium by aiding its uptake and distribution within the host.

epithelial barrier; mucosal immunology; tight junctions; host defense

SYMPTOMS OF Salmonella enterica serovar Typhimurium (S. typhimurium) infection manifest within 48 h after ingestion of contaminated food or water and include nausea, vomiting, and acute, mostly self-limiting, diarrhea. Following ingestion, the pathogen is able to colonize the intestinal tract and penetrate the intestinal epithelium to ultimately gain access to systemic sites, such as the liver and spleen, through lymphatic and blood circulation. S. typhimurium invasion genes are necessary for bacterial invasion of intestinal epithelial cells and are thought to allow Salmonella to enter and cross the intestinal epithelium during infection by manipulation of the host cytoskeleton (9, 11). At present, the capacity of S. typhimurium to enter nonphagocytic cell types is thought to be critical for accessing deeper tissues during infection. Indeed, the ability of S. typhimurium to breach the intestinal barrier during infection has been correlated with its ability to invade cultured epithelial cells (10). This correlation has provided an important model for studying the molecular interactions between Salmonella and nonphagocytic host cells. This is also the most widely accepted mechanism for how Salmonella overcomes the intestinal barrier.

More recent findings suggest that there are multiple pathways by which S. typhimurium can gain access to the circulation. CD18 expressing phagocytes can take up S. typhimurium and transport the bacteria from the intestinal tract to the blood stream, spleen, and liver (46). In addition, CD18 positive dendritic cells (DC) have been demonstrated to take S. typhimurium across the intestinal mucosa and possibly carry the bacteria to the spleen and liver (37). In mice, S. typhimurium is rapidly detected in lamina propria DCs, which may acquire Salmonella through transepithelial dendrites from the subepithelial space, or apoptotic intestinal epithelial cells (32). Also, in Peyer’s patches, DCs rapidly acquire S. typhimurium and initiate mucosal immune responses (39). Other studies have demonstrated that S. typhimurium can affect the integrity of the epithelial barrier (2, 6, 19, 34).

Modulation of epithelial permeability properties is one of the common outcomes of bacterial infection of epithelial monolayers in vitro, and the in vivo correlates of these effects may induce or amplify diarrhea. The composition of the proteins that make up the tight junctional complex are highly dynamic, and several bacterial pathogens have been able to modulate epithelial tight junctions to their own advantage. However, the direct interaction of a bacterial virulence factor on component proteins of the tight junction has been proposed only in a few instances (40). The assembly of the tight junctional complex underlies the development of intestinal barrier integrity and serves to strictly partition the apical and basolateral plasma membrane domains of the cells (13). A key functional attribute of the epithelial cell tight junction also limits the permeation of substances and translocation of bacteria via the paracellular space (24). Such tight junctional complexes are macromolecular assemblies of proteins that form contiguous rings at the apices of the epithelial cells. In addition to peripheral membrane proteins, these proteins consist of several cytoplasmic proteins, such as the zonula occludens (ZO)-1, ZO-2, and ZO-3.
isogenic hilA S. typhimurium that invades epithelial cells. S. typhimurium strain VV341 is a streptomycin-resistant wild-type strain and was used in the study (23). T84 cell monolayers were drained of media containing fresh HBSS/H9262. Either wild-type S. typhimurium, VV341, or E. coli F-18 was added to the basal surface of the Transwell filter. Bacterial aliquots of either wild-type S. typhimurium, VV341, or E. coli F-18 were administered individually to the apical surface of T84 cell monolayers at an inoculation ratio (MOI) or equivalent of 400 bacteria/epithelial cells and incubated for 120 min at 37°C. TER was measured at the time points indicated.

Assessment of T84 Cell Monolayer Permeability

The permeability of the T84 cell monolayer was evaluated by using the paracellular flux marker [3H]inulin, which has a Stokes radius of 10.5 Å and a molecular weight of 5.2 kDa. Briefly, the apical surface of T84 cell monolayers was infected with either wild-type S. typhimurium, VV341, or E. coli F-18 at an MOI of 400 for 120 min at 37°C. After the infection with bacteria, 2.5 μCi/ml of [3H]inulin were added to the apical membrane compartment of the Transwell filter. The percent of [3H]inulin that traversed the monolayer from the apical to basolateral direction was determined after 3 h at 37°C. Counts per minute of radioactivity were determined in a β-scintillation counter (Beckman LS1801).

Bacterial Translocation Through Preinfected T84 Intestinal Epithelial Monolayers

Bacterial aliquots of either wild-type S. typhimurium, VV341, or E. coli F-18 were administered individually to the apical surface of T84 cell monolayers at a MOI of 400 and subsequently incubated for 120 min at 37°C. After extensive washing, the extracellular bacteria were killed by treatment of the monolayer with gentamicin (480 μg/ml gentamicin for 60 min at 37°C). This initial infection by these bacteria will be referred to as the “priming infection.” Following the priming infection, the cell monolayers were transferred into wells containing fresh HBSS+. Either wild-type S. typhimurium, VV341, or healthy individual and is completely avirulent (28). Nonagitated microaerophilic bacterial cultures were prepared by inoculating 10 ml of Luria-Bertani (LB) broth with 0.01 ml of a stationary phase culture, followed by overnight incubation at 37°C, as described previously (25). Bacterial supernatants of S. typhimurium were obtained by incubating bacteria at 37°C for 1 h in HBSS containing Ca2+ and Mg2+ supplemented with 10 mM HEPES, pH 7.4 (Sigma Chemical), referred to as HBSS+, at concentrations equivalent to the infection dose mentioned above before centrifugation (8,500 g, 10 min). Heat-killed bacteria were prepared by boiling in a water bath for 30 min (the killing efficiency was above 99.99%). The heat-killed bacteria and the bacterial supernatant were used individually at a concentrations equivalent to those used for the living bacteria (see above). LPS derived from S. typhimurium by phenol extraction (Sigma, St. Louis, MO) was diluted in HBSS+ to a final concentration of 10 μg/ml and added in a similar fashion to that described for S. typhimurium.

Cell Viability

The viability of the T84 cells before and after bacterial infection with either wild-type S. typhimurium, S. typhimurium strain VV341, or E. coli F-18 was assessed by Trypan blue dye exclusion and lactate dehydrogenase (LDH) secretion assays. The LDH secretion was measured from the cellular supernatant by a commercially available LDH assay kit (Sigma Chemical) and carried out as described by the manufacturer’s instructions. Lysis of the cells with 1% Triton X-100 served as a positive control. All conditions except the Triton X-100 treated monolayers showed a Trypan blue dye exclusion rate of >95%.

Measurement of TER

TER was used to monitor the integrity of the epithelial monolayer with a Volt-ohm meter (World Precision Instruments, New Haven, CT). Only those monolayers that exhibited a TER of 800–1,500 Ω-cm² were considered to have an appropriate barrier function and were used in the study (23). T84 cell monolayers were drained of media and gently washed with HBSS+. Unless otherwise indicated, the monolayers were equilibrated at 37°C for 25 min with HBSS+. The bacterial suspension, heat-killed bacteria, bacterial supernatant, or LPS were administered to the apical surface of T84 cell monolayers at an inoculation ratio (MOI) or equivalent of 400 bacteria/epithelial cells and incubated for 120 min at 37°C. TER was measured at the time points indicated.

Materials and Methods

Cell Culture

T84 intestinal epithelial cells (passages 46–66) were grown in a 1:1 mixture of Dulbecco-Vogt modified Eagle’s medium and Ham’s F-12 nutrient mixture supplemented with 15 mM HEPES (pH 7.5), 14 mM NaHCO3, 40 μg/ml penicillin, 8 μg/ml streptomycin, 8 μg/ml ampicillin, and 6% fetal bovine serum. Monolayers were grown on 5-μm pore size collagen-coated permeable polycarbonated filters (Costar, Cambridge, MA) of 0.33 cm² for transmigration and translocation assays and 4.7 cm² for cell harvest and immunoblotting and utilized 7 and 21 days, respectively, after plating, as described previously (23). In some experiments, T84 cells were grown on four-well slide culture chambers (Nunc, Naperville, IL) and were used 2 days after confluence. Conventional monolayers were used to study the translocation of bacteria, and inverted monolayers were used to perform experiments that involved polymorphonuclear leukocyte (PMN) transepithelial migration (36). A steady-state transepithelial cell resistance (TER) of ~800–1,500 Ω-cm² was reached in all monolayers used.

Bacterial Strains and Growth Conditions

S. typhimurium SL1344 is a streptomycin-resistant wild-type strain that invades epithelial cells. S. typhimurium strain VV341 is an isogenic hila mutant of SL1344, which is invasion defective. Escherichia coli F-18 is a normal intestinal flora isolate harvested from a healthy individual and is completely avirulent (28). Nonagitated microaerophilic bacterial cultures were prepared by inoculating 10 ml of Luria-Bertani (LB) broth with 0.01 ml of a stationary phase culture, followed by overnight incubation at 37°C, as described previously (25). Bacterial supernatants of S. typhimurium were obtained by incubating bacteria at 37°C for 1 h in HBSS containing Ca2+ and Mg2+ supplemented with 10 mM HEPES, pH 7.4 (Sigma Chemical), referred to as HBSS+, at concentrations equivalent to the infection dose mentioned above before centrifugation (8,500 g, 10 min). Heat-killed bacteria were prepared by boiling in a water bath for 30 min (the killing efficiency was above 99.99%). The heat-killed bacteria and the bacterial supernatant were used individually at a concentrations equivalent to those used for the living bacteria (see above). LPS derived from S. typhimurium by phenol extraction (Sigma, St. Louis, MO) was diluted in HBSS+ to a final concentration of 10 μg/ml and added in a similar fashion to that described for S. typhimurium.

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E. coli F-18 were then added to the apical side of the epithelial monolayers for 60 min at an MOI of 400 to establish a secondary infection. After the secondary infection, buffer from the basolateral compartment of the epithelial cell monolayer was collected and plated for colony-forming units to assess the numbers of bacteria that traversed the cell monolayer from the second infection. Parallel experiments controlled for the potential translocation of bacteria from the primary infection and the data presented are normalized accordingly. All conditions were tested in triplicate and the experiment was performed at least twice.

PMN Transepithelial Migration

The physiologically directed (basolateral to apical) PMN transepithelial migration in response to a bacterial stimulus has been previously detailed (36). Briefly, human PMNs were isolated from normal volunteers by gelatin sedimentation, as described elsewhere (17) and 10^6 PMNs were added to the basolateral compartment of the T84 cell monolayer. Induction of PMN transepithelial migration was induced in one of two ways. For physiological induction, T84 cell monolayers either were infected from the apical membrane domain with S. typhimurium at a MOI of 400 for 2 h at 37°C or were directly stimulated by adding imposed gradients of the chemotaxant N-formylmethionin-leucyl-phenyalanine (fMLP) across the monolayer at a concentration of 10 nM (25, 26). In a subset of experiments, PMN transepithelial migration was measured by inducing imposed gradients of fMLP across T84 cell monolayers previously infected with S. typhimurium. After each transmigration assay, nonadherent PMN were extensively washed from the basolateral surface of the cell culture inserts, and PMN cell equivalents, estimated from a standard curve, were assessed as the number of PMN that had completely traversed the cell culture insert (i.e., into the basolateral reservoir). Neutrophil isolation was limited to repetitive donations by 10 different donors over the course of these experiments. Because of variation in donor neutrophils, the data were analyzed within an individual experiment and not between experiments. However, the overall trends associated with these data are reproducible between experiments.

Western Blot Analysis

S. typhimurium were added to the apical surface of inverted T84 cell monolayers at a MOI of 400 for 120 min at 37°C. Immediately before and during the infection period, T84 cell monolayers were harvested at 0, 60, and 120 min and Triton X-100-soluble and -insoluble protein fractions were prepared as described previously (33). Briefly, monolayers were harvested on ice in 350 μl of lysis buffer [1% Triton X-100, 100 mM NaCl, 10 mM HEPES, 2 mM EDTA, 4 mM Na3VO4, 40 mM NaF 200 mM PMSF, and a protease inhibitor cocktail (Complete Mini, Roche Molecular Biochemicals, Mannheim, Germany)]. The lysates were centrifuged (14,000 g for 30 min at 4°C) and the supernatant suspension, representing the Triton X-insoluble fraction, was collected. All samples were used immediately or stored at −80°C. The protein concentration of each sample was quantified by the Bradford method (Bio-Rad, Hercules, CA). Samples were electrophoresed through a 4–20% gradient SDS polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After 1-h blocking (Tris-buffered saline, 0.1% Tween 20, 1% BSA), the blots were incubated overnight at 4°C with primary antibodies diluted in blocking buffer. After being washed, the membrane was incubated for 1 h at room temperature with the appropriate secondary antibody diluted in blocking buffer. The hybridized band was detected by chemiluminescence using an ECL kit (Amersham) according to the manufacturer’s instructions. In some instances, immunoblots were stripped with 62.5 mM Tris (pH 6.8) 2% SDS containing 10 mM 2-β-mercaptoethanol.

Immunostaining and Confocal Microscopy

The S. typhimurium strains (wild type and VV341) or E. coli F-18 were individually administered to the apical surface of T84 cell monolayers at an MOI of 400 at 37°C. At the indicated time intervals, cells were washed three times with Dulbecco’s PBS (Life Technologies, Gaithersburg, MD) and fixed in acetone for 1 min at −20°C. Thereafter, the cells were blocked with a 1:20 dilution of normal goat serum diluted in PBS (blocking solution) for 30 min at 20°C. Primary antibodies were diluted in the blocking solution and were incubated for 1 h at room temperature (1:200 for anti-E-cadherin antibody and 1:100 for anti-ZO-1, ZO-2, claudin-1, and occludin antibodies). After three washes with PBS, the monolayers were incubated with FITC-conjugated secondary antibody (1:250 in blocking solution) at room temperature for 1 h without light. Monolayers were then analyzed with an AX-70 Olympus microscope and a confocal immunofluorescent microscope (Bio-Rad).

Antibodies

Rabbit polyclonal antibodies against ZO-1, ZO-2, occludin, and claudin-1 were purchased from Zymed Laboratories (San Francisco, CA). Mouse monoclonal anti-E-cadherin antibody was obtained from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were obtained from Amersham (Arlington Heights, IL).

Drug Treatment

Chelerythrine chloride. Where indicated, T84 cells were preincubated with the PKC inhibitor chelerythrine chloride (ChCl) (Biomol, Plymouth, PA) at final concentrations of 5 μM. Bacterial infection of T84 cell monolayers was carried out in the continued presence of ChCl.

EDTA treatment. In a subset of experiments, T84 cell monolayers were treated with the Ca2+ chelator EDTA to open the tight junctions. This procedure was performed as described by Parkos et al. (35). Briefly, T84 monolayers were grown on permeable filter supports, washed free of media, and exposed to HBSS− (calcium and magnesium free) containing 2 mM EDTA. The monolayers were then incubated for 12 min at 37°C and were subsequently washed in HBSS+ to replete calcium. This treatment resulted in a loss of electrical resistance over several hours (>3 h) as reported previously (35).

Protease inhibitors. A subset of experiments was also carried out with the protease inhibitors antipain (100 μM), AEBSF (100 μM), pepstatin (1 μM) (Sigma Chemical), and a protease inhibitor cocktail (Complete Mini, Roche Molecular Biochemicals, Mannheim, Germany) prepared according to the manufacturer’s instructions.

Statistics

All results are expressed as means ± SD of an individual experiment performed in triplicate. P values were calculated according to Student’s t-test, and values <0.05 were considered statistically significant.

RESULTS

Salmonella Effects on Epithelial Barrier Function

To investigate the effect of S. typhimurium on the integrity of the epithelial barrier established by T84 cell monolayers, we first measured the TER under various conditions. As shown in Fig. 1, we found that wild-type S. typhimurium induced a decrease in the TER of T84 cell monolayers as early as 30 min
postinfection. This decrease continued throughout the 2 h infection period and after 120 min the TER of the S. typhimurium infected monolayer was 9 ± 1.5% of the preinfection value. However, incubation of the apical surface of the epithelial monolayer with either S. typhimurium supernatant, heat-killed S. typhimurium, or LPS did not adversely influence the TER during the same time course. To control for the effects of nonspecific protease activity on the TER of T84 cell monolayers, the addition of protease inhibitors (antipain, AEBSF, pepstatin, and a protease inhibitor cocktail) failed to prevent the loss of TER during infection with S. typhimurium (data not shown). We next determined whether the effects seen with wild-type S. typhimurium on T84 cell TER were specific. Thus we compared wild-type S. typhimurium to its isogenic derivative hilA mutant VV341 and the nonpathogenic human intestinal isolate, E. coli F-18 (Fig. 1). Unlike wild-type S. typhimurium, we found that E. coli F-18 did not adversely affect the T84 cell electrical resistances and in fact exhibited a significant increase in TER at early times points (30 min postinfection). However, the S. typhimurium mutant strain VV341 induced a progressive decrease in T84 cell TER but not as dramatic as wild-type S. typhimurium (TER value of 50% of the preinfection value was recorded after 120 min for VV431).

We next sought to determine whether such alterations induced by wild-type S. typhimurium with regard to TER correlated with changes in epithelial cell permeability and used [3H]inulin as a marker for the paracellular flux studies (Fig. 2). Under conditions of wild-type S. typhimurium infection, we found that inulin was fivefold more effective in paracellular flux studies (Fig. 2). The infected monolayer with either the Salmonella hilA− mutant (VV341) showed slower kinetics on the ability to decrease TER compared with the wild-type strain. Infection with the nonpathogenic Escherichia coli F-18 strain lead to an increase in TER, which was only statistically significant at 30 min postinfection. Data are expressed as means ± SD for triplicate samples for all conditions tested and represent 1 of at least 3 experiments performed. *P < 0.01; **P < 0.05.

S. typhimurium Modulates Tight Junctional Components in Polarized Model Intestinal Epithelia

Since wild-type S. typhimurium had an adverse affect on epithelial barrier function with respect to electrical resistance and permeability, we next analyzed whether infection of T84 cell monolayers with this pathogen could influence the expression and/or intracellular distribution of the major proteins, which comprise the tight junctional complex.

Claudin-1. As shown in Fig. 3A, we determined that the transmembrane tight junction protein claudin-1 was redistributed between the Triton X-100-insoluble and -soluble fractions. Specifically, in the Triton X-100-insoluble fraction we observed a slight decrease of claudin-1 expression 60 and 120 min after S. typhimurium wild-type infection, whereas cell monolayers either infected with E. coli F-18 or treated with only buffer failed to exhibit a modulation in claudin-1 expression. To verify these observations at the cellular level, we examined claudin-1 expression by immunofluorescence 120 min after S. typhimurium infection (detailed in MATERIALS AND METHODS). Although en face immunofluorescence imaging showed a moderate increase in claudin-1 expression in the area near tight junction cell contacts compared with the buffer-treated control (Fig. 4), the E. coli image did not reveal a shift in claudin-1 expression along the apical to basolateral membrane axis.

Occludin. Infection of the epithelial monolayer with S. typhimurium resulted in a dephosphorylation of hyperphosphorylated occludin in the insoluble fraction (molecular weight

Figs. 1 and 2. Effect of S. typhimurium on transepithelial resistance (TER). The apical infection of a T84 cell monolayer with S. typhimurium wild type (SL1344) leads to a rapid decrease of TER. This effect is only marginally altered by the PKC inhibitor chelerythrine chloride (ChCl). The infection of the monolayer with the Salmonella hilA− mutant (VV341) showed slower kinetics on the ability to decrease TER compared with the wild-type strain. Infection with the nonpathogenic Escherichia coli F-18 strain lead to an increase in TER, which was only statistically significant at 30 min postinfection. Data are expressed as means ± SD for triplicate samples for all conditions tested and represent 1 of at least 3 experiments performed. *P < 0.01; **P < 0.05.
band between 72 and 79 kDa). Correspondingly, the nonphosphorylated occludin band (between 65 and 71 kDa) shown in Fig. 3A increased in intensity in the insoluble fraction following 120 min of infection. We did not observe a difference in the phosphorylation state of occludin in the soluble fraction. In addition, the \textit{S. typhimurium hilA} mutant VV341 exhibited a pattern of occludin dephosphorylation, although the kinetics were not as rapid as in the wild type. Furthermore, the specificity of this result was demonstrated by the observation that \textit{E. coli} F-18 failed to modulate either the expression of occludin or its phosphorylation state (Fig. 3A). Subsequent analysis by immunohistochemistry revealed that, following an infection with \textit{S. typhimurium}, occludin was removed from the epithelial tight junctions and redistributed into small cytoplasmic vesicles. We could also detect occludin in the lateral cell membrane compartment below tight junctions, indicating a vertical shift of this protein along the lateral membrane (Fig. 4).

\textit{ZO-1}. In the control, T84 cell monolayers grown on filter supports the hypothesis that the tight junction protein ZO-1 was only detected in Triton X-100-insoluble fraction (Fig. 3A). However, following an infection of these monolayers with \textit{S. typhimurium} over a 120-min time course we determined that expression of ZO-1 was dramatically decreased in a progressive fashion. Despite the fact that the expression of ZO-1 was considerably reduced at 120 min after \textit{S. typhimurium} infection in the Triton X-100-insoluble fraction, this protein did not appear to be redistributed to the Triton X-100-soluble fraction. By contrast, infection of T84 cell monolayers with the \textit{S. typhimurium} \textit{hilA} mutant did not affect the expression of ZO-1. This observation suggests that ZO-1 regulation may be dependent on the \textit{Salmonella} pathogenicity island 1 (SPI1). The specificity of this result was demonstrated by \textit{E. coli} F-18, which failed to modify the expression and/or distribution of ZO-1 in polarized cell monolayers. Although Western blot analysis clearly demonstrates that ZO-1 disappeared from the Triton X-100-insoluble protein fraction 120 min after infection with \textit{S. typhimurium}, this protein was unexpectedly observed in the areas of cellular contacts by immunohistochemistry, albeit to a decreased level (Fig. 4; see DISCUSSION for explanation).
pointed out that the regulation seen in the *S. typhimurium* hilA− mutant was just slightly less pronounced than that observed for wild-type *S. typhimurium*. Not surprisingly, the negative control strain *E. coli* F-18 failed to influence the expression or intracellular distribution of either ZO-2 or E-cadherin (Fig. 3A). This finding was verified by immunohistochemistry. Under conditions of wild-type *S. typhimurium* infection, ZO-2 was clearly recruited from the cytosol to a more pronounced membrane location (Fig. 4). Moreover, *E. coli* immunofluorescent image analysis further showed that expression of ZO-2 extended further down the basolateral membrane axis compared with the buffer control. Unfortunately, immunofluorescent staining of the monolayer with E-cadherin antibodies produced inconclusive results.

The above alterations of epithelial tight junction in response to *S. typhimurium* infection were not the result of cell death. Both Trypan blue exclusion and LDH release assays confirmed the viability of the T84 cells before and after *S. typhimurium* infection. Specifically, the LDH secretion from both infected and uninfected cell monolayers were in the range of 66–100 units/ml, whereas the lysed cells had a LDH concentration of 266 units/ml in the supernatant.

*Salmonella* Infection Promotes Bacterial Translocation Through an Epithelial Monolayer

Having demonstrated that *S. typhimurium* can influence the integrity of the epithelial barrier and can specifically modulate the expression state and distribution of intercellular tight junction proteins, we next sought to determine the physiological consequences of these events. We first examined whether a priming infection by wild-type *S. typhimurium* could promote bacterial traversal of the intestinal barrier following a second wave of infection. In this experiment, polarized monolayers of T84 cells were infected with *S. typhimurium* at the apical membrane domain for 120 min followed by gentamicin treatment to kill the extracellular bacteria. This initial infection was challenged by a second wave of infection by *S. typhimurium* introduced again from the apical surface. As shown in Fig. 5, we found that priming the T84 cells with wild-type *S. typhimurium* greatly enhanced the ability of *S. typhimurium* (from the secondary infection) to gain access to the basolateral compartment, resulting in a 10-fold increase compared with uninfected control monolayers. Consistent with this observation, a priming infection with wild-type *S. typhimurium* significantly increased the translocation of the nonpathogenic *E. coli* F-18 when this strain was used as the secondary infection (Fig. 5). Taken together, these data indicate that the breaches induced by wild-type *S. typhimurium* in model intestinal epithelia are nonspecific with regard to bacterial passage. It is interesting to note that although the *S. typhimurium* hilA− mutant induced a 50% decrease in T84 cell TER but had no effect on paracellular inulin flux. The priming infection with this strain only tripled the number of wild-type *S. typhimurium*, which were able to traverse the monolayer (down nearly threefold compared with its wild-type parent). Not surprisingly, when the T84 epithelial cell monolayers were primed with *E. coli* F-18, this organism failed to promote bacterial translocation established by a secondary infection with either wild-type *S. typhimurium* or *E. coli* F-18 (Fig. 5). These data are consistent with the fact that *E. coli* F-18 did not alter T84

**Fig. 4.** *S. typhimurium* modulates epithelial tight junction. Immunostaining for tight junction proteins after apical stimulation with *S. typhimurium* for 2 h. A, C, E, and G: En face immunostaining. B, D, F, and H: confocal microscopy, original magnification ×100.
cell monolayer TER or permeability or influence the expression or distribution of the T84 cell tight junctional proteins. In addition, we treated T84 cells with the Ca\(^{2+}\) chelator EDTA as another means of altering TER (35; described in MATERIALS AND METHODS). As shown in Fig. 5, we found that although EDTA treatment of T84 polarized cell monolayers led to a significant increase in bacterial translocation for both \(S.\) typhimurium and \(E.\) coli F-18, the enteric pathogen \(S.\) typhimurium was more proficient in its ability to translocate across intestinal epithelium. Because a common feature of gut inflammation is an increase in epithelial barrier permeability, these data are consistent with current dogma suggesting that perturbations in epithelial barrier function can lead to bacterial translocation of both pathogenic and nonpathogenic enteric microorganisms.

**Effect of Tight Junction Alterations on PMN Transepithelial Migration**

Intestinal infection of Salmonella is known to lead to a substantial mucosal inflammatory response (10). This is histologically characterized by the transmigration of PMN across the intestinal epithelium in the basolateral to apical direction and typically results in the formation of intestinal lesions and crypt abscesses. Intestinal epithelial cells respond to \(S.\) typhimurium by releasing distinctive proinflammatory chemottractants, such as IL-8 and hepoxilin \(\Lambda_3\) (formerly known as PEEC), which sequentially orchestrate PMN movement across the intestinal epithelium (26, 27, 31). On the basis of these important observations, we next sought to determine whether alterations of the epithelial cell tight junction created by infection with \(S.\) typhimurium could facilitate the movement of PMNs across the monolayer.

To analyze whether alterations of the tight junctional complex had an impact on the physical movement of PMN across T84 cell monolayers, we prevented the ability of \(S.\) typhimurium-stimulated epithelial cells to activate a PKC signal transduction pathway required for the release of hepoxilin \(\Lambda_3\) (22, 31). We found that the PKC inhibitor ChCl had no effect on epithelial barrier function or on the expression and distribution of the tight junctional proteins (Fig. 3B). In addition, as shown in Fig. 1, ChCl did not interfere with the ability of \(S.\) typhimurium to induce alterations in T84 cell TER or modulate the expression or cellular distribution of the tight junctional proteins, with the exception of ZO-1. In this regard, we found that stimulation of the epithelial cell monolayer with wild-type \(S.\) typhimurium in the presence of ChCl failed to modulate the expression of ZO-1. These results suggest that elements of PKC activation may be used by Salmonella to alter ZO-1 expression.

Control experiments were performed next to confirm that ChCl completely inhibited \(S.\) typhimurium-induced PMN migration across a model intestinal monolayer but had no effect on PMN migration to imposed gradients of the chemoattractant fMLP (Fig. 6). Having established these parameters, we next determined whether altering tight junctional components could facilitate PMNs across the epithelial monolayer. Thus PMN transmigration was initiated by adding imposed gradients of fMLP across T84 cell monolayers, which had previously been infected with \(S.\) typhimurium in the presence of ChCl. These conditions allowed for the physical assessment of PMN movement across epithelial cell monolayers. We found that the potential of PMN to transmigrate across epithelial cell monolayers was increased 1.75 times compared with monolayers that were not infected with \(S.\) typhimurium but were otherwise treated in the same manner (Fig. 6). To control for the effect of ChCl in this set of experiments another pan-PKC inhibitor, staurosporine, was used, and this inhibitor behaved in the same manner as ChCl (data not shown). Additionally, as a negative control, we verified that infection of the T84 cell monolayers with nonpathogenic \(E.\) coli F-18 did not enhance fMLP-induced PMN migration. These results indicate that observed effects of \(S.\) typhimurium on epithelial cell barrier function are specific and are not the result of random PMN movement (Fig. 6). Under similar conditions we found that infection of T84 cell monolayers with the \(S.\) typhimurium hila\(^{-}\) mutant, VV341, failed to enhance PMN migration, presumably because of its inability to fully modulate the epithelial barrier. Collectively, these findings indicate that alterations of the intercellular junctions caused by \(S.\) typhimurium in epithelial cell monolayers can facilitate PMN movement across the intestinal epithelium.

**DISCUSSION**

In this study, we investigated the mechanisms underlying the effects of \(S.\) typhimurium on epithelial barrier function. A number of enteric pathogens are known to perturb the intestinal
epithelial barrier and impact TER or paracellular permeability, most often with an alteration in the arrangement of tight junctional component proteins by mechanisms, which may be unique for different pathogen (40). For example, Clostridium difficile toxins A and B enhance the permeability by disrupting actin microfilaments within the perijunctional ring (16), and enteropathogenic E. coli disrupt the epithelial barrier by the phosphorylation of myosin light chains (49). With respect to S. typhimurium, in vitro models of infection have revealed an alteration of epithelial permeability, which in the case of the kidney epithelial cell line MDCK involves rapid changes in both tight junction permeability and transcellular conductance. In particular, S. typhimurium infection of MDCK cell monolayers induced contraction of the perijunctional actin ring with a corresponding increase in paracellular permeability (19). It was originally proposed that contraction of the perijunctional actinomyosin ring might physically disrupt the tight junctions, since tension generated through actinomyosin had been implicated in the modulation of tight junction permeability (5, 19). However, recent studies indicate that perijunctional contraction is not required for Salmonella-induced tight junction dysfunction (20). The Salmonella effector protein SigD (also called SopB), which is encoded in SPI-1, is able to elicit a reduction in epithelial barrier function, perhaps via activation of PKC (2). Also, the effector proteins SopB, SopE, SopE2, and SipA as well as the activation of geranylgeranylated proteins are necessary to disrupt the epithelial barrier and alter the distribution at least some tight junction proteins (3, 44). The goal of the present study was to further elucidate the mechanisms underlying the effects of S. typhimurium on epithelial barrier function.

S. typhimurium rapidly induced the dephosphorylation of occludin within human intestinal epithelial cells. Occludin is a membrane-spanning protein that forms two extracellular loops and is thought to play a primary role in the maintenance of the epithelial barrier. The breakdown of the tight junctional barrier has been associated with the dephosphorylation of occludin by enteropathogenic E. coli and Shigella flexneri (38, 41, 48). The hilA mutant isoforms of occludin induced the dephosphorylation of occludin; however, the rate of dephosphorylation was slower compared with its wild-type parent. The regulation of occludin by either wild-type S. typhimurium or the hilA mutant correlated well with the disruption of the epithelial barrier. Since the association of occludin with tight junctions requires its phosphorylation, our data indicate that SPI1 may play a role in the regulation of the epithelial barrier function during S. typhimurium invasion by regulating the availability of functional occludin. hilA promotes the expression of the effector proteins SopB, SopE, SopE2, and SipA, which are essential for the disruption of the epithelial barrier by Salmonella (3).

ZO-1 establishes the linkage between transmembrane proteins and the cytoskeleton. Previous studies have shown that infection of MDCK II cell monolayers with S. typhimurium led to the clustering of f-actin, E-cadherin, and ZO-1 at the apical membrane domain (20). Here, we have found that wild-type S. typhimurium, but not the hilA mutant, was able to rapidly decrease ZO-1 from tight junction associated Triton X-100-insoluble protein fractions. Therefore, the ability of S. typhimurium to downregulate ZO-1 was dependent on the type III secretion system encoded by SPI1. Furthermore, the regulation of ZO-1 by invasive S. typhimurium was found to be dependent on the activation of the PKC pathway, similar to a recent finding that PKC signaling regulates Clostridium difficile toxin A-mediated paracellular permeability changes and ZO-1 translocation (4).

We found that both wild-type and hilA mutant strains of S. typhimurium induced the rapid recruitment of ZO-2 and E-cadherin from the cytosolic protein pool into the Triton X-100-insoluble fraction. ZO-2 has been shown to be immunoprecipitated as a stable complex with ZO-1 (47). However, we have recently determined that the functional compartmentalization of ZO-1 and ZO-2 may be distinctly regulated in intestinal epithelial cells infected with Shigella flexneri (38). Further experiments will be necessary to determine whether the recruitment of ZO-2 is an effort by the epithelium to compensate for a temporal loss of ZO-1.

Claudins share a similar membrane topology with occludin, and there is accumulating evidence to suggest that claudins constitute the backbone of tight junction protein strands (45).
These tight junction proteins are thought to play a role in the regulation of paracellular electrolyte transport (29). We found only a weak modulation of claudin-1 expression in tight junction following infection with *S. typhimurium*. This is in contrast to *S. flexneri*, which is able to specifically target and temporally regulate claudin-1 from tight junctions (38). The only other organism known to specifically target claudins is *Clostridium perfringens* via its enterotoxin (42). The specific SPI1 secreted effectors responsible for the ability of *S. typhimurium* to compromise the epithelial barrier are currently under investigation (3). Such studies propose that tight junction disruption by *S. typhimurium* is the result of the effect of Rho GTPases on the actin cytoskeleton. In this study, over the course of a *S. typhimurium* infection with mutant strains ΔSipA, ΔSopB, ΔSipA/SopB, or ΔSopE/E2 decreased ZO-1 and occludin expression occurred to the same extent as the wild type. However, infection with either a ΔSopB/E/E2 or a ΔSipA/SopE/E2 mutant did not appreciably perturb the expression levels of ZO-1 or occludin. On the basis of these observations, Boyle et al. (3) conclude that whereas SopB, SopE, SopE2, and SipA are the effectors underlying the disruption of tight junction structure and function by *S. typhimurium*, SipA and SopB were found to be involved but these effectors are insufficient for barrier disruption.

Having determined whether infection with *S. typhimurium* could regulate the molecular composition of the tight junction, we further considered whether such modifications could influence bacterial translocation and PMN movement across model intestinal epithelium. We found that, following an initial priming infection with *S. typhimurium*, a secondary infection established by either *S. typhimurium*, hilA−, or the nonpathogen *E. coli* F-18 led to a considerable increase in bacterial translocation across the intestinal monolayer. In vivo, this may pave the path to the enhanced access of *Salmonella* to the lamina propria itself, leading to the invasion or uptake into migratory phagocytes. A recent study indicates that under metabolic stress intestinal epithelial cells increase their endocytic activity, resulting in a microtubule-microfilament-dependent internalization and transcytosis of nonpathogenic *E. coli* (31a). Although we cannot definitively rule out a transcytotic mechanism, on the basis of increases in endocytic activity we are less likely to favor this possibility since transcytosis of *E. coli* to the basolateral compartment was assessed after 24 h (31a), whereas in our model system we observed bacterial translocation much more rapidly (1 h), suggesting the bacteria are traversing via a paracellular route. Nevertheless, increased microbial influx most likely enhances local inflammatory responses observed in vivo during *S. typhimurium* infection. In the case of *S. typhimurium*, the acute response to gastrointestinal infection is characterized by an initial accumulation of PMN in the lamina propria followed by transepithelial migration. Among the events stimulated by this specific interaction is the epithelial release of potent PMN chemoattractants, such as IL-8 and heparin A3, which act in concert to guide PMN into the intestinal lumen (26, 27, 31). In addition to this primary mechanism of PMN movement across epithelial monolayers, results from this study identify a secondary means by which *S. typhimurium* can directly influence PMN-epithelial cell interactions.

As our previous reported studies indicated that infection with *S. typhimurium* did not influence TER it would appear that this study contradicts our prior findings (25). However, there are key differences between this report and our earlier work that could account for such discrepancies. First, the *S. typhimurium* background strains used in these two studies are different. Our original work employed the 14028 background *S. typhimurium* strain (25), whereas the studies in the present report used the SL1344 background strain. Since the SL1344 strain induces a more robust response in the ability to induce PMN transepithelial migration compared with the 14028 strain (19.3 ± 2.30 and 11.24 ± 1.53 × 10^4 PMN cell equivalent/ml for SL1344 and 14028 infected monolayers, respectively) it is quite possible that such differences in the virulence phenotype could explain, in part, differences at the level of the TER. Moreover, in the previous experiments the TER measurements of the monolayers selected for infection had a significantly lower initial TER compared with initial TER measurements of the monolayers selected for the present studies. Thus any effect mediated by *S. typhimurium* on TER within 120 min may have been masked by a lower TER before infection. Nevertheless, our present findings are consistent with reports indicating that infection of model intestinal epithelium wild-type *S. typhimurium* leads to a fall in TER (2, 3).

In sum, our findings demonstrate that invasive *S. typhimurium* can modulate epithelial tight junction integrity enough to allow the physical movement of PMN across the intestinal monolayer. This may be the direct result of the regulation of tight junction-associated proteins since NH2-terminal modifications of occludin enhanced PMN movement across epithelial cell monolayers (18). Together, we demonstrate that the invasion of polarized human intestinal epithelial cells by *S. typhimurium* is associated with rapid molecular changes in the composition of tight junctional complexes leading to the loss of barrier function. The disruption of epithelial barrier integrity by *Salmonella* has pathological consequences by enhancing bacterial translocation across the epithelial barrier and fosters disease pathology by promoting migration of PMNs.

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REFERENCES


GROUNDBED S, Finlay BB.

Gumbiner BM.


McCormick BA, Hofman PM, Kim J, Carnes DK, Miller SI, Madara JL.


