Unmasking of Intestinal Epithelial Lateral Membrane $\beta 1$ Integrin Consequent to Transepithelial Neutrophil Migration In Vitro Facilitates *inv*-Mediated Invasion by *Yersinia pseudotuberculosis*

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Idiopathic intestinal disease states characterized by active inflammation associated with transepithelial migration of neutrophils may, paradoxically, be associated with an increased risk of infection by enteric pathogens. Although the specific ligands with which various intestinal pathogens associate remain largely unknown, it is thought that many reside on the basolateral membrane. For example, $\beta 1$ integrin, a basolateral membrane protein, mediates the specific interaction between epithelial cells and the *inv* gene product (invasin) on the surface of *Yersinia pseudotuberculosis*. Our observations indicate that neutrophil migration across model T84 cell intestinal epithelia produced transient separation of epithelial cells at sites of neutrophil migration, resulting in microdiscontinuities that remained unsealed for several hours. We hypothesized that such sites of microdiscontinuities would yield a potential route for luminal pathogens to gain access to basolateral ligands and, thus, provide a window of risk for enteric infection. The surface biotinylation and fluorescence localization studies reported here revealed that, as in natural intestinal epithelia, $\beta 1$ integrin was strictly polarized to the basolateral membrane in confluent T84 monolayers. However, the transient microdiscontinuities resulting from neutrophil migration permitted access to $\beta 1$ integrin from the apical reservoir. Coincident with such basolateral exposure of $\beta 1$ integrin, monolayers became susceptible to invasion by *Y. pseudotuberculosis*. Fluorescence localization indicated that *Y. pseudotuberculosis* selectively associated with monolayers at sites where small discontinuities resulting from neutrophil transmigration were found. An increased risk for *Y. pseudotuberculosis* infection was specifically related to exposure of $\beta 1$ integrin (normally concealed by tight junctions) to the apical compartment, as *Y. pseudotuberculosis* cells lacking the *inv* gene were unable to invade following neutrophil transepithelial migration. Following closure of the microdiscontinuities associated with neutrophil migration, a small pool of $\beta 1$ integrin remained apically localized, presumably due to incomplete repolarization. However, this small apical pool of $\beta 1$ integrin was insufficient to support a detectable increased risk of *Yersinia* infection. Together, these observations indicate that by transiently perturbing monolayer continuity, neutrophil transepithelial migration is associated with a window of risk in which luminal pathogens can access basolateral ligands such as $\beta 1$ integrin.

The apical and basolateral surfaces of columnar epithelial cells are biochemically distinct (4, 38). Such surface polarization provides for the requisite asymmetrical distribution of membrane proteins necessary to support vectorial transepithelial transport processes. The circumferential apical tight junctions which separate the apical and basolateral domains of adjoining cells not only assist in maintenance of epithelial cell surface polarity ("fence" function) but also provide high-resistance intercellular seals ("gate" function) (3, 10). Thus, tight junctions, by isolating basolaterally restricted proteins from luminal bacteria, also serve to restrict the repertoire of potential interactions which may occur between luminal bacteria and the epithelial cell surface. For example, available evidence suggests that the intestinal pathogen *Shigella flexneri* preferentially enters intestinal epithelial cells by means of a basolateral ligand(s). Although the specific epithelial ligand(s) for this pathogen remains unidentified, perturbations which likely pro-

vide access to the basolateral surface enhance invasion by *S. flexneri* and thus support the basolateral entry hypothesis (27, 34). Better understood in this regard is the enteric pathogen *Yersinia pseudotuberculosis*. Invasin, the *Y. pseudotuberculosis* outer membrane protein product of the *inv* gene, specifically recognizes many $\beta 1$ integrins on eucaryotic cells, and the resulting invasin- $\beta 1$ integrin-based interactions are required for this pathogen's ability to invade host cells (14). While much of the data supporting $\beta 1$ integrin targeting by *Y. pseudotuberculosis* come from nonintestinal, nonpolarized cell types, it is anticipated that naturally polarized target cells, intestinal epithelial cells, would interact with this pathogen in a similar fashion. However, $\beta 1$ integrins on columnar epithelia are basolaterally polarized, indicating that the epithelial ligand for the *Y. pseudotuberculosis* invasin protein is normally separated from luminal pathogens by the gate and fence functions (10) of intercellular tight junctions.

How, then, might luminal organisms with putative basolateral ligands interact with polarized intestinal epithelia? One widely suggested possibility is that transcytosis across specialized M cells which overlie Peyer's patches may ultimately provide for interaction with basolateral components of neighboring cells, permitting subsequent lateral cell-to-cell spread in

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the basolateral compartment (18, 36, 41). However, while this route is likely an important one, the area of the mucosal surface lined by M cells represents a minor portion (far less than 1%) of the epithelial surface area (42). A second potential means by which luminal pathogens might access basolateral ligands is transient perturbation of the intercellular tight junction, thus permitting luminal organisms focal access to the basolateral domain. Given the vast surface area covered by the non-M-cell population, such events, even if rare, might significantly contribute to initiation of these infections.

Active inflammation of the intestine is defined by transepithelial migration of neutrophils. We have utilized a model of the human intestinal epithelial cell line T84 and human peripheral blood neutrophils to recapitulate this event (25, 29, 31). This polarized intestinal epithelial model has been used to examine mechanisms governing neutrophil adherence to intestinal epithelia (30) and neutrophil-induced alterations in intestinal epithelial transport (23, 40), and the findings generated by this model have correctly anticipated similar mechanisms in natural intestinal epithelia (28, 32, 40). Such studies have also demonstrated that during active transepithelial migration of neutrophils, tight junction integrity, as assessed by biophysical and permeability characteristics, is reversibly lost. Interestingly, diseases such as idiopathic inflammatory bowel disease, in which transepithelial migration of neutrophils occurs inappropriately (in the absence of an identifiable pathogen) and, once established, often continues unabated, are characterized by an increased risk of infection with intestinal pathogens.

Given the disrupting effects of transepithelial neutrophil migration on the ability of intestinal epithelial tight junctions to maintain a separation between the lumen and intercellular spaces, we investigated whether such events lead to a window of risk for interaction between luminal pathogens and known basolateral ligands on the epithelial cell. Since *Y. pseudotuberculosis* has a well-defined, basolaterally polarized ligand (β1 integrin), we used this organism to address this question. β1 integrin was documented to be appropriately localized to the basolateral domain of polarized T84 cells, and subsequent neutrophil transepithelial migration was found to lead to foci in which epithelial cells were parted from neighbors, resulting in focal availability to luminal elements of basolateral ligands. Such sites served as specific targets for entry of *Y. pseudotuberculosis* into epithelial cells in a manner which was dependent on bacterial expression of the *inv* gene—the protein product of which interacts specifically with β1 integrin. Together, these observations suggest that focal perturbations of tight junctions induced by normal pathophysiological events, such as neutrophil transmigration (8), are sufficient to permit exposure of basolateral ligands for luminal pathogens such as *Y. pseudotuberculosis*. As a consequence, epithelial inflammation produces a window of risk for host invasion by pathogens which recognize lateral ligands normally “masked” by intact tight junctions. Additionally, since previous data have defined the mechanism by which *Y. pseudotuberculosis* interacts with β1 integrin in unpolarized nonintestinal target cells (13–15), these data also confirm that the invasins-β1 paradigm applies to the natural initial target cell of this pathogen.

MATERIALS AND METHODS

Tissue culture. T84 intestinal epithelial cells (passages 70 to 95) were grown and maintained as confluent monolayers on collagen-coated permeable supports (28, 33) with recently detailed modifications (21, 31). T84 cells were grown as monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle medium and Ham's F-12 medium supplemented with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (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. Monolayers were grown on 0.33-cm² ring-supported

polycarbonate filters (Costar, Cambridge, Mass.) and utilized 6 to 14 days after plating, as described previously (21, 31). Steady-state resistance was reached in 4 to 6 days, with variability largely related to cell passage number. Monolayers received one weekly feeding following initial plating. Inverted monolayers, used to study transmigration of polymorphonuclear neutrophils (PMNs) in the physiological basolateral-to-apical direction, were constructed as described before (21, 28, 31, 33).

Electrophysiology. To assess currents, transepithelial potentials, and resistance, a commercial voltage clamp (Bioengineering Department, University of Iowa) was employed and interfaced with an equilibrated pair of calomel electrodes submerged in saturated KCl along with a pair of Ag-AgCl electrodes submerged in Hanks balanced salt solution (HBSS). Agar bridges were used to interface the electrode with the solutions on either side of the monolayers (one calomel and one Ag-AgCl electrode in each well), and measurements of short-circuit current and resistance were made as detailed elsewhere (22).

Neutrophil transmigration. The physiologically directed (basolateral-to-apical) PMN transepithelial migration assay has been previously described (33). Neutrophil transmigration experiments were performed at 37°C on 0.33- and 5-cm² inserts with human neutrophils isolated as previously described (33). PMNs were isolated from healthy human volunteers of both sexes as described elsewhere (11, 33). Once isolated, the PMNs were suspended in modified HBSS (without Ca²⁺ and Mg²⁺, with 10 mM HEPES [pH 7.4; Sigma Chemical Co.]) at a concentration of 5×10^7 /ml. We added 2×10^6 PMNs to the 0.33-cm² inserts and 3.5×10^7 PMNs to the 5-cm² inserts. Transmigration of PMNs was initiated by applying 1.0 μM *N*-formyl-1-methionyl-1-leucyl-1-phenylalanine (fMLP) to the lower reservoir and incubating it for 15 min to allow a transepithelial chemotactic gradient to form prior to the addition of PMNs. Transepithelial resistances and short-circuit currents were then monitored over the 120-min course of transmigration. After 120 min, both 0.33- and 5-cm² inserts were washed with warm HBSS and transferred into new wells with fresh medium in the upper and lower reservoirs. Transepithelial resistances were then monitored until recovery of baseline resistances.

Localization of β1 by confocal fluorescence microscopy. To localize β1 integrin, monolayers were fixed in 3.75% paraformaldehyde (10 min, 20°C) and then incubated in HBSS containing 5% normal goat serum with (to detect apical and basolateral integrin) or without (to detect apical integrin and basolateral integrin at sites where confluence was disrupted) permeabilization. Anti-β1 integrin in 5% normal goat serum-HBSS was added to the apical surface of the monolayers and allowed to bind for 1 h (20°C). After washing, monolayers were incubated for 1 h (20°C) with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Stained monolayers were then mounted and visualized by using a Bio-Rad MRC confocal fluorescence microscope (Bio-Rad, Richmond, Calif.) as previously described (16). In control localization experiments, fixed monolayers were then permeabilized in 0.2% Triton X-100 followed by staining as described above. In control monolayers, basolateral β1 integrin was detected only by an apically applied antibody following permeabilization. An irrelevant antibody failed to stain (data not shown).

Biotinylation and immunoprecipitation. Surface biotinylation of T84 membranes was carried out as previously described (17). Briefly, T84 cells grown on 5-cm² permeable supports were cooled to 4°C and washed with cold HBSS. Apical or basolateral surfaces were selectively biotinylated by adding 0.5 mg of biotin sulfo-*N*-hydroxysuccinimide ester (Pierce Biochemicals, Rockford, Ill.) per ml in HBSS to the inner (apical) or outer (basolateral) chamber and incubated for 20 min at 4°C. The reaction was quenched by incubating monolayers in 50 mM NH₄Cl in HBSS for 20 min at 4°C and then washing them several times with ice-cold HBSS. Monolayers were then briefly washed in Ca²⁺- and Mg²⁺-free HBSS and scraped from filters into 10 mM HEPES (pH 7.4)–3.5 mM MgCl₂–150 mM NaCl–1 mM phenylmethylsulfonyl fluoride–1 mM benzamide–10 mM chymostatin–10 mM leupeptin–1 mM pepstatin–1 mM bestatin. For each condition, T84 cells from three monolayers (15 cm², 1.5×10^7) were pooled and washed. The washed cell pellets were then solubilized in 600 μl of buffer containing 2% Triton X-100 (at 4°C). Insoluble cellular debris was sedimented by centrifugation (10,000 × *g* for 10 min) and then precleared for 1 h with protein G-Sepharose. The precleared extract was incubated overnight with 10 μl of an anti-β1 monoclonal antibody (mouse monoclonal antibody C4P10 [Telios, Inc.]), followed by protein G-Sepharose. Immunoprecipitates were washed with 10 mM NaH₂PO₄ (pH 7.4)–1% Nonidet P-40–0.4 M NaCl–2 mM EDTA–0.1 M NaF–1 mM benzamide–10 mM chymostatin–10 mM leupeptin–1 mM pepstatin–1 mM bestatin) and then denatured in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting by standard protocols. Protein surface labelled with biotin was visualized after incubation with peroxidase-conjugated streptavidin by enhanced chemiluminescence in accordance with the manufacturer's instructions (Amersham, Inc., Buckinghamshire, United Kingdom). Films were scanned with a 300A scanning densitometer from Molecular Dynamics, Sunnyvale, Calif. Band densities were semiquantitatively analyzed with ImageQuant Software (Molecular Dynamics).

Bacterial strains. *Y. pseudotuberculosis* YPIII (P[−]) (*inv*⁺; pIBI cured) and YP232 (*inv*-lacking; Kan^r pIBI cured) were a kind gift of Ralph Isberg (Department of Microbiology, Tufts University Medical School) and were grown and handled as previously described (12). Both of these yersinia strains have been cured of a 70-kbp virulence plasmid, pIBI, which encodes additional factors that promote bacterial penetration of cultured mammalian cells. *Salmonella typhi*-

murium χ 3306 is a nalidixic acid-resistant (*gryA1816*) strain derived from *S. typhimurium* SR-11 (9).

Media. Luria broth (LB) was made as described by Revel (37). L agar is LB containing 12 g of Bacto Agar (Difco Laboratories, Detroit, Mich.) per liter. MacConkey agar (Difco Laboratories) was prepared in accordance with package instructions.

Growth conditions. The *Y. pseudotuberculosis* strains were grown in LB with constant agitation at 28°C. Bacteria from such cultures were in the mid- to late-logarithmic phase of growth and correlated with 1×10^8 to 2×10^8 CFU/ml routinely. CFU were determined by diluting and plating onto MacConkey agar medium (Difco Laboratories). For specific details of bacterial preparation for each experiment, see below.

Yersinia invasion assays. To analyze invasion of epithelial monolayers by *Y. pseudotuberculosis*, strains were grown overnight at 28°C, diluted 1:50, and then grown for an additional 3 to 4 h at 28°C (12). The bacterial suspension was washed twice in HBBS, and 20- μ l aliquots (equivalent to approximately 4×10^6 yersiniae per monolayer and five bacteria per epithelial cell) were added to the apical interface of polarized T84 monolayers, which were then incubated at 37°C for 90 min. The monolayers were washed twice to remove any nonadherent bacteria. Separation of invaded versus attached organisms was performed by the gentamicin sensitivity assay (12, 19). Monolayers were lysed with 1% Triton X-100 for 10 min at 4°C and placed in LB, and serial dilutions and plating onto MacConkey agar plates permitted subsequent determination of the number of CFU per sample (25). For fluorescence detection of epithelial cell-bound *Y. pseudotuberculosis*, strains were labeled with rhodamine (Molecular Probes). *Y. pseudotuberculosis* cells were grown to the log phase, washed twice in a sterile saline solution, and resuspended (28°C for 2 h) in saline containing rhodamine at 0.3 mg ml⁻¹. They were then used following exhaustive washing in saline. This treatment was found not to influence binding to epithelia (data not shown). Monolayers to which these bacteria attached were fixed for 20 min at 4°C in 3.7% paraformaldehyde, extracted in acetone for 4 min at -20°C, and counterstained for F actin with FITC-phalloidin (39). Monolayers were examined by confocal microscopy. In a subset of experiments, epithelial monolayer and *Y. pseudotuberculosis* assays were performed as outlined above, and Ca²⁺ depletion, which reveals lateral ligands recognizing cells added to the luminal compartment was performed as previously described (30). Briefly, confluent T84 cell monolayers grown in a conventional fashion (apical surface facing upwards) on 0.33-cm² permeable filters were transiently exposed to 2 mM EDTA. After 12 min (at 37°C), the monolayers were washed in HBSS to replenish Ca²⁺. Transsepithelial cell resistance was measured throughout to ensure ablation of barrier function. Transient exposure of confluent T84 monolayers to EDTA permits access of the paracellular space from the apical surface. Tight-junction integrity is Ca²⁺ dependent, and treatment of epithelial monolayers with EDTA opens tight junctions, resulting in epithelial cell depolarization (24, 30, 35). After such EDTA exposure, monolayers were used to assess bacterial invasion profiles. *S. typhimurium* use, growth, and assays were exactly as previously described (25).

Neutrophil transepithelial migration coupled to yersinia invasion. Steps describing the protocol for neutrophil transepithelial migration coupled to yersinia invasion are outlined in Fig. 1. Briefly, following posttransmigration events, polarized T84 cell monolayers were washed in HBSS buffer to remove nonassociated PMN, returned to fresh wells containing HBSS buffer, and incubated for 1 h at 37°C. *Y. pseudotuberculosis* strains prepared as described above were then added to the apical surface of inverted T84 monolayers, which were incubated for 90 min at 37°C as described above for *Yersinia* invasion assays. Nonadherent bacteria were removed by washing with ice-cold HBSS. The washed monolayers were then treated with 50 μ g of gentamicin per ml for 90 min, and after extensive washes, the T84 monolayers were lysed with Triton X-100 and cells lysates were diluted and plated onto MacConkey agar as described above. Negative control experiments were performed in the absence of neutrophil transmigration to the chemoattractant stimulus fMLP (100 nM). All experiments were performed in a 37°C room. In the subset of experiments detailing post wound closure studies, neutrophils were applied as described above and incubated for 90 min at 37°C. However, following neutrophil transmigration, the monolayers were washed in sterile HBSS, returned to fresh medium, and incubated for 20 h. This allowed time to reestablish the integrity of the monolayer, as assessed by monitoring of transepithelial resistances.

RESULTS

Transepithelial migration of neutrophils transiently exposes basolateral β 1 integrin. Polarized monolayers of a human intestinal epithelial cell line (T84) display phenotypic and biophysical properties of natural intestinal crypt epithelia, including vectorial ion transport processes comparable to those of the intestinal crypt, the ability to interact with transmigrating neutrophils in an appropriate ligand-restricted manner, and maintenance of strict apical (i.e., luminal) and basolateral surface polarity (1, 2, 16, 33). Thus, given the potential disrupting effects of transepithelial migration of neutrophils on

FIG. 1. (A) PMN transepithelial migration. Confluent T84 cell monolayers grown in an inverted fashion (basolateral surface facing upward) on 0.33-cm² permeable filters (before utilization in assays, the monolayers were washed and maintained in HBSS physiologic buffer [pH 7.4]) were subjected to neutrophil transepithelial migration in the basolateral-to-apical direction via the influence of an imposed gradient of fMLP (10^{-7} M). Nonadherent neutrophils were removed by extensive washing in HBSS buffer and returned to fresh wells containing HBSS for equilibration. (B) *Yersinia* invasion. *Y. pseudotuberculosis* was then added to the apical surface of these inverted monolayers, as previously described in detail (25), which were then housed in a moist chamber for 90 min at 37°C. (C) Assessment of invasion profile. Nonadherent bacteria were removed by washing in HBSS, and the colonized, polarized cell monolayers were treated with 50 μ g of the prokaryote-specific antibiotic gentamicin per ml. Gentamicin, an aminoglycoside antibiotic, does not permeate eukaryotic plasma membranes and is therefore cytolytic only to extracellular populations of bacteria while intracellular bacterial populations remain viable (19). After treatment, monolayers were washed, lysed with Triton X-100, diluted, and plated onto MacConkey agar. TJ's, tight junctions.

the ability of intestinal epithelial tight junctions to maintain separation between the lumen and intercellular spaces (29), we investigated whether such events lead to a window of risk for interaction between luminal pathogens and basolateral ligands on epithelial cells. Immunofluorescence of one such ligand, β 1 integrin (Fig. 2A and B), revealed selective expression on the basolateral cell surface in control detergent-permeabilized monolayers. Since intercellular tight junctions of T84 monolayers restrict passive movement of molecules between cells, addition of anti- β 1 to the apical surface of T84 monolayers did not label the basolateral cell surface unless sites of junctional microdiscontinuities were produced by neutrophil migration across T84 monolayers in response to the chemotactic peptide fMLP (29). To locate epithelial microdiscontinuities produced by transepithelial migration of neutrophils, the apical surface of monolayers was briefly exposed to anti- β 1 antibody following neutrophil transmigration. Subsequent visualization of this antibody by fluorescence permitted identification of sites at

FIG. 2. Localization of $\beta 1$ integrin on intestinal epithelial (T84) cell monolayers and access to $\beta 1$ integrin from the luminal compartment following transepithelial migration of neutrophils. (A and B) In control permeabilized monolayers, immunofluorescence localization (A, en face; B, transverse, x-z section) demonstrates the strict basolateral polarization of total $\beta 1$ integrin (closed arrowheads) with no detectable staining on the apical membrane (open arrowhead). Since tight junctions are intact in control monolayers, monolayers must be permeabilized so that an apically applied antibody can access the basolateral compartment. (C) Following neutrophil migration across epithelia, microdiscontinuities (asterisks) develop which can be highlighted by transient apical exposure of unpermeabilized monolayers to an antibody to $\beta 1$ integrin. This approach highlights the $\beta 1$ integrin exposure to the luminal compartment at these sites (solid arrowheads highlight exposed basolateral membranes which surround holes [asterisks] in the monolayer). (D) Eighteen hours following neutrophil transmigration, microdiscontinuities have resealed (and monolayers are confluent, as judged by biophysical criteria [see text]). However, at sites where discontinuities have apparently closed, a focal pool of apically displaced $\beta 1$ integrin can be detected (arrowhead). (Scale bar; 20 μm).

which neutrophil transmigration had led to microdiscontinuities such that an apical antibody could recognize $\beta 1$ integrin. These epithelial discontinuities represent foci of high-density neutrophil transmigration (28, 29). Transepithelial migration of $(5.80 \pm 0.60) \times 10^4$ neutrophils across epithelial monolayers produced multifocal microdiscontinuities 30 to 100 μm in monolayers (Fig. 2C) paralleled by decreases in transepithelial resistance to passive ion flow ($1,627 \pm 28$ to $525 \pm 24 \Omega \cdot \text{cm}^2$ [$P < 0.01$]). Lateral epithelial cell surfaces immediately adjacent to the microdiscontinuities were strongly labeled with apically applied anti- $\beta 1$ antibody, presumably due to the disruption of epithelial tight junctions at these sites (Fig. 2). Structurally defined resealing of epithelial monolayers occurred by 18 h subsequent to neutrophil transmigration (Fig. 2D), although rare sites of apical $\beta 1$ staining were observed, and was paralleled by a return of transepithelial resistance to values indicative of biophysically defined confluency ($1,172 \pm 130 \Omega \cdot \text{cm}^2$) (20, 21).

Biochemical analyses of selective apical or basolateral surface expression of $\beta 1$ integrin by biotinylation techniques confirmed that the expression of $\beta 1$ integrin (apparent molecular mass, 120 kDa) is normally restricted to the basolateral cell surface (Fig. 3). However, in a wounded monolayer, transient microdiscontinuities resulting from neutrophil migration permitted access to $\beta 1$ from the apical reservoir, demonstrated by a dramatic increase in the $\beta 1$ signal. Further, in agreement with fluorescence localization studies (Fig. 2), apical biotinylation of the epithelial cell surface following resealing of microdiscontinuities (18 h) detected only a small pool of apically displaced $\beta 1$ (Fig. 3). This small, apically displaced pool of $\beta 1$

integrin appeared to represent incomplete recovery of normal surface polarity at sites where microdiscontinuities had closed.

Luminal *Y. pseudotuberculosis* invades enterocytes via luminally exposed $\beta 1$ integrin. Although several luminal pathogens are thought to interact preferentially with basolateral ligands on the epithelial cell surface (14, 27, 34), the molecular nature of such procaryotic-eucaryotic ligand-ligand interactions remains obscure. One exception to this is *Y. pseudotuberculosis*, a human intestinal pathogen which has been shown to interact specifically with $\beta 1$ integrins (α_3 , α_4 , α_5 , and α_6) of eucaryotic cells via the bacterial outer membrane protein invasins, the product of the *inv* gene (13–15). The findings in Fig. 2 indicated that a pool of $\beta 1$ integrin might be accessible from the luminal surface following transepithelial migration of neutrophils. Thus, we next determined whether the luminal access to $\beta 1$ integrins afforded by the transient epithelial microdiscontinuities arising from neutrophil transmigration was sufficient to convert such monolayers to a *Y. pseudotuberculosis* invasion-sensitive phenotype. As shown in Fig. 4A, epithelial T84 monolayers became sensitive to invasion by *Y. pseudotuberculosis*

FIG. 3. Selective surface biotinylation of $\beta 1$ integrin. (A) Localization of $\beta 1$ integrin in intact polarized monolayers of T84 intestinal epithelial cells was resolved by immunoprecipitation of $\beta 1$ integrin, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 to 16% gradient gel), and detection of the biotinylation signal as detailed in Materials and Methods. Expression of $\beta 1$ (indicated by the solid arrow at 120 kDa) in control monolayers is selective to the basolateral (BL) surface versus the apical (AP) surface. In the postwounded state, in which microdiscontinuities have been introduced into the monolayer as a result of neutrophil transmigration, there is a dramatic increase in the $\beta 1$ signal. Consistent with the findings shown in Fig. 2D, following closure of microdiscontinuities during overnight (O.N.) recovery, only a small pool of apically displaced $\beta 1$ integrin can be detected. Data are representative of at least two experiments, and results were obtained by scanning with Adobe Photoshop software. (B) Densitometry scan. For semiquantitative analysis, the relative band densities of $\beta 1$ integrin for the control monolayer apical and basolateral surfaces and monolayers displaying postwound and overnight recovery phenotypes paralleled the results shown in panel A. Data represent means $\pm$ standard deviations of two experiments.

FIG. 4. Microdiscontinuities in epithelial monolayers produced by neutrophil transmigration induces monolayer sensitivity to invasion by *Y. pseudotuberculosis* as a function of $\beta 1$ integrin exposure to the lumen. (A) Invasion of epithelial monolayers by *inv*⁺ *Y. pseudotuberculosis* [YPIII (P-); closed bars] (12) is enhanced following transepithelial migration of neutrophils. In contrast, *inv* mutant *Y. pseudotuberculosis* (YP232; open bars) (12), which selectively lacks the gene product which recognizes $\beta 1$ integrin as a ligand, shows identical characteristics of interaction (attachment and invasion) with epithelial monolayers in the presence or absence of epithelial discontinuities produced by neutrophil transepithelial migration (*, $P < 0.05$). The left halves of panels B and C demonstrate the filamentous actin staining pattern of T84 cell monolayers stained with FITC-phalloidin under low-power magnification. Asterisks in both B and C represent immunolocalization of microdiscontinuities (corresponding sites in the right half of panel B are also shown by asterisks). The right halves of panels B and C illustrate the interaction of rhodamine-stained *Y. pseudotuberculosis* with the same T84 monolayers depicted on the left. Specifically, only *inv*⁺ *Y. pseudotuberculosis* selectively associated with lateral membranes of epithelial cells at sites of discontinuity (arrowheads in panel B). No such association was seen with *inv* mutant *Y. pseudotuberculosis* (right half of panel C).

following transepithelial migration of neutrophils. To determine whether such sensitivity to invasion by *Y. pseudotuberculosis* was specifically due to accessibility of epithelial $\beta 1$ integrin as a ligand, experiments were performed with a *Y. pseudotuberculosis* strain in which the *inv* gene was selectively deleted (Fig. 4A). As shown in Fig. 4A, conversion of the epithelial monolayer to a *Y. pseudotuberculosis* invasion-sensitive phenotype by neutrophil migration was dependent on expression of the *inv* gene and therefore dependent on $\beta 1$ -mediated *Y. pseudotuberculosis*-epithelium interactions. Thus, the transient microdiscontinuities resulting from neutrophil migration permitted access to $\beta 1$ integrin from the apical reservoir. As a result, an increased risk for invasin-expressing *Y. pseudotuberculosis* infection was attributable to $\beta 1$ (normally concealed by tight junctions) exposure to the apical compartment, while *Y. pseudotuberculosis* lacking the *inv* gene was unable to invade the intestinal epithelium following neutrophil transepithelial migration. Imaging studies were next performed to localize the site of *inv* gene-mediated *Y. pseudotuberculosis* association with monolayers containing neutrophil-induced microdiscontinuities. As described in Materials and Methods, such studies consisted of a double-labeling procedure in which the T84 monolayers were labeled with FITC-phalloidin to stain specifically for F-actin, while *Y. pseudotuberculosis* was directly pre-labeled with rhodamine. As illustrated in Fig. 4B and C, an en face (x-y) confocal image in a plane corresponding to the apical surface of a wounded T84 monolayer depicts the hexagonal staining pattern of F-actin networks typically displayed in the apex of columnar epithelial cells. Gaps representing the sites where previously neighboring cells have been parted by trans-migrating neutrophils, and which correspond to the images seen in Fig. 2, are readily visible. Immunofluorescent labeling of *Y. pseudotuberculosis* within such monolayers revealed that the bacteria associated specifically with epithelial cells at sites distinguished by microdiscontinuities (Fig. 4B) and showed that *Y. pseudotuberculosis* expression of *inv* is required for this specific association (Fig. 4B and C). In contrast, after microdiscontinuities had resealed (18 h; see above), monolayer sensitivity to invasion by *Y. pseudotuberculosis* was lost ($0.30\% \pm 0.07\%$ versus $0.26\% \pm 0.09\%$ of the original inoculum invaded, representing three bacteria internalized per 10 epithelial cells; control and 18 h resealed monolayers were not significantly different). Efficiency of internalization into polarized T84 cell monolayers has been previously shown to be 10- to 50-fold less than that reported for nonpolarized, nonintestinal epithelial cell lines (26). To test whether *Y. pseudotuberculosis* binding to monolayers with epithelial discontinuities secondary to neutrophil transmigration was, in part, due to binding to residual neutrophils entrapped in the monolayer, an alternate double-staining protocol was performed. Neutrophils were labeled with anti-CD11b (OKM1) and identified with a fluorescein-labelled secondary antibody, while rhodamine conjugation of *Y. pseudotuberculosis* was again used to localize the bacteria. Such studies demonstrated only rare colocalization of *Y. pseudotuberculosis* with residual neutrophils (data not shown) and emphasize the suggestion that in a wounded epithelium, basolateral ligands become available such that only invasin-expressing *Y. pseudotuberculosis* is able to bind exposed ligands. Thus, taken together, such data support the hypothesis that creation of sites of microdiscontinuities by neutrophil transepithelial migration yields a potential route for luminal pathogens to gain access to basolateral ligands and, thus, provides a window of risk for enteric infection.

The above observations further suggest that binding of $\beta 1$ integrin by luminal *Y. pseudotuberculosis* at sites where neutrophil transmigration has transiently disrupted epithelial tight

junctions is the basis for conversion of epithelial monolayers from a *Y. pseudotuberculosis* invasion-resistant state to a *Y. pseudotuberculosis* invasion-sensitive state. To further confirm this hypothesis, experiments were performed with transient extracellular Ca^{2+} depletion (see Materials and Methods), a maneuver which disrupts intercellular tight junctions and permits access of lumenally applied cells to basolateral surface ligands, resulting in epithelial cell depolarization while preserving the columnar architecture of the intestinal epithelial cells (30). Under these conditions, the resulting exposure of lateral ligands to the apical compartment converted epithelial monolayers to a *Y. pseudotuberculosis*-sensitive state. Furthermore, conversion to this sensitive state specifically relied on expression of the *inv* gene by *Y. pseudotuberculosis* (Fig. 5A). Thus, Ca^{2+} depletion, similar to neutrophil transepithelial migration, did not substantially enhance attachment of *inv*⁺ *Y. pseudotuberculosis* but greatly aided in internalization. In contrast, both attachment and internalization profiles of *inv* mutant *Y. pseudotuberculosis* cells were unaffected by Ca^{2+} depletion (data not shown). Such data support the proposal that pathophysiological events which lead to disruption of tight junctions can enhance internalization of *Y. pseudotuberculosis* and such enhancement is strictly *inv* dependent.

Although it is presumed that many intestinal pathogens initiate interactions with the host via basolateral surface ligands, there are also examples of enteric pathogen interactions with the apical epithelial membrane. For example, *S. typhimurium*, another human intestinal pathogen, appears to interact with and enter intestinal epithelial cells directly via the apical membrane, without requiring basolateral ligand availability (5–7, 25). Hence, to verify that the observed increase in *Y. pseudotuberculosis* internalization seen posttransmigration of neutrophils did not extend to bacteria which entered via apical mechanisms, we examined *S. typhimurium* associations with monolayers during this state. Accordingly, we found that *S. typhimurium* adhesion to and invasion of T84 epithelial monolayers was not influenced by the presence of the microdiscontinuities produced by neutrophil migration across the intestinal epithelium (Fig. 5B). Such data indicate that indiscriminate internalization of surface-bound bacteria does not account for the enhanced internalization observed with *inv*⁺ *Y. pseudotuberculosis* following neutrophil transmigration.

DISCUSSION

The establishment of tight junctions between columnar epithelial cells both assists in maintaining the lipid and protein asymmetry between the apical and basolateral cell membranes (fence function) and regulates the flow of hydrophilic molecules through the spaces between cells (gate function) (3, 10). Since the luminal spaces lined by such epithelia are in contact with the external environment, these fence and gate functions prevent the interaction of environmental pathogens with basolateral cell surfaces that may harbor ligands recognized by environmental pathogens (14, 27, 34). During inflammation at mucosal surfaces, neutrophils migrate across epithelial tight junctions and into spaces such as the lumen of the digestive tract. The data reported here suggest that the presence of epithelial microdiscontinuities, created by the events which characterize epithelial inflammation, affords a window of opportunity for luminal pathogens to recognize ligands on the lateral surface of epithelial cells. Under conditions in which intact tight junctions, by means of their gate function, separate apically positioned yersiniae from basolaterally polarized β1 integrin, these pathogens are unable to invade intestinal epithelial cells. However, as neighboring epithelial cells become

FIG. 5. Exposure of lateral ligands converts epithelia to the *Y. pseudotuberculosis* invasion-sensitive phenotype but does not influence epithelial associations of pathogens (*S. typhimurium*) which do not require lateral ligands to invade epithelial cells. (A) Diffuse disruption of tight junctions was obtained by means of brief EDTA exposure and also resulted in conversion of epithelia to the *Y. pseudotuberculosis* invasion-sensitive state. Such conversion was dependent on expression of the *inv* gene (*, $P < 0.001$). Tx, Triton X-100. (B) The presence of microdiscontinuities induced by neutrophil transmigration does not influence the associations between epithelia and a pathogen (*S. typhimurium*) which enters via apical, rather than basolateral, ligands (no statistically significant difference).

separated from one another (loss of junctional gate) by the force of transmigrating neutrophils, yersiniae gain access to the unmasked basolateral β1 ligand, which results in subsequent invasion of epithelial cells. The finding that enhanced invasion following neutrophil transepithelial migration does not occur with an isogenic strain in which the *inv* gene is deleted strongly supports the interpretation that invasion of epithelia by yersiniae is selectively promoted by unmasking of the invasin ligand β1 integrin. Further substantiating this notion, following closure of such microdiscontinuities associated with neutrophil transmigration, the invasion capacity of *Y. pseudotuberculosis* was significantly diminished.

The above outlined observations suggest that by transiently perturbing epithelial monolayer continuity, neutrophil transepithelial migration is associated with a window of risk in which luminal pathogens can access basolateral ligands such as $\beta 1$ integrins. Since a small detectable apical pool of $\beta 1$ integrin was shown to persist at sites where microdiscontinuities had resealed, it was possible that resealed monolayers might exhibit residual sensitivity to invasion by *Y. pseudotuberculosis* for a time following closure, that is, until complete lateral repolarization of this integrin was achieved. However, analyses of such monolayers early after resealing revealed no detectable sensitivity to *Y. pseudotuberculosis*. Possible explanations for these observations include the following: (i) the increment of invasion after closure of microdiscontinuities was too small to detect, (ii) the density of $\beta 1$ integrin at these sites was too low to support multivalent interactions that are likely necessary for efficient invasion, or (iii) the distinctive cytoskeletal cortex underlying the apical membrane is incompatible with $\beta 1$ integrin-mediated invasion by this organism. Nonetheless, such findings indicate that the window of opportunity for invasion by luminal *Y. pseudotuberculosis* ends with closure of the microdiscontinuities produced by neutrophil transmigration. In addition, presumptive exposure of speculative basolateral ligands for *S. flexneri*, associated with neutrophil transepithelial migration, has also been hypothesized to facilitate invasion of intestinal epithelia by this microorganism (34).

The data presented in this report, however, provide important potential mechanistic insight into clinical observations indicating that diseases in which transepithelial migration of neutrophils occurs inappropriately and, once established, continues unabated also carry an increased risk of infection by enteric pathogens. Transepithelial migration of neutrophils in idiopathic conditions such as chronic ulcerative colitis and Crohn's disease often occurs inappropriately, fails to remit, and ultimately produces abundant epithelial microdiscontinuities similar to those shown here (8). Such discontinuities are apt to exist for several hours following neutrophil transmigration and may serve as sites for establishment of invasion by pathogens which selectively recognize lateral ligands on epithelia. Thus, as with many responses of the human immune system, inappropriate expression-regulation of this immune response may enhance the risk of inflammatory disease.

Our findings indicate that the normal gate and fence functions of intercellular tight junctions of epithelia combine to mask lateral ligands recognized by environmental pathogens. In so doing, tight junctions restrict the repertoire of available ligands capable of facilitating interactions between environmental pathogens and columnar epithelial cells. The lateral ligand masking function of tight junctions thus represents a characteristic of this structure, in addition to its known gate and fence functions, which likely is particularly important to pathobiology of disease produced by environmental pathogens.

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