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Neutrophil-mediated epithelial injury during transmigrating: role of elastase

HEDY H. GINZBERG,¹ VERA CHERAPANOV,² QIN DONG,² ANDRE CANTIN,³
CHRISTOPHER A. G. McCULLOCH,⁴ PATRICK T. SHANNON,⁵ AND GREGORY P. DOWNEY²
¹Department of Pediatrics, Division of Gastroenterology and Nutrition and ²Department of
Medicine, Division of Respiratory, The University of Toronto, Toronto, Ontario, Canada M5S 1A8;
³Department of Medicine, Division of Respiratory, The University of Sherbrooke,
Sherbrooke, Quebec, J1H 5N4; ⁴Faculty of Dentistry, University of Toronto, CIHR Group
in Periodontal Physiology and ⁵Department of Pathobiology and Laboratory Medicine,
The University of Toronto, Toronto, Ontario, Canada M5S 1A8

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Ginzberg, Hedy H., Vera Cherapanov, Qin Dong, Andre Cantin, Christopher A. G. McCulloch, Patrick T. Shannon, and Gregory P. Downey. Neutrophil-mediated epithelial injury during transmigrating: role of elastase. *Am J Physiol Gastrointest Liver Physiol* 281: G705–G717, 2001.—Neutrophil-mediated injury to gut epithelium may lead to disruption of the epithelial barrier function with consequent organ dysfunction, but the mechanisms of this are incompletely characterized. Because the epithelial apical junctional complex, comprised of tight and adherens junctions, is responsible in part for this barrier function, we investigated the effects of neutrophil transmigration on these structures. Using a colonic epithelial cell line, we observed that neutrophils migrating across cell monolayers formed clusters that were associated with focal epithelial cell loss and the creation of circular defects within the monolayer. The loss of epithelial cells was partly attributable to neutrophil-derived proteases, likely elastase, because it was prevented by elastase inhibitors. Spatially delimited disruption of epithelial junctional complexes with focal loss of E-cadherin, β -catenin, and zonula occludens 1 was observed adjacent to clusters of transmigrating neutrophils. During neutrophil transmigration, fragments of E-cadherin were released into the apical supernatant, and inhibitors of neutrophil elastase prevented this proteolytic degradation. Addition of purified leukocyte elastase also resulted in release of E-cadherin fragments, but only after opening of tight junctions. Taken together, these data demonstrate that neutrophil-derived proteases can mediate spatially delimited disruption of epithelial apical junctions during transmigration. These processes may contribute to epithelial loss and disruption of epithelial barrier function in inflammatory diseases.

leukocyte; inflammation; adherens junctions; adhesion molecules; epithelium; inflammatory mediators; tight junctions

EPITHELIAL CELLS FORM A SELECTIVE barrier and are responsible for the active transport of fluid, ions, and small molecules. Epithelial injury can occur as a consequence of inflammation when injury to the epithe-

lium of organs (e.g., intestine, kidney, and lung) is associated with extensive infiltration by neutrophils (4, 21, 41). In these disorders, unregulated movement of fluid and electrolytes across the injured epithelium can lead to edema of the organ (e.g., pulmonary edema) or loss of fluid and electrolytes from the body (e.g., diarrhea).

Polarized epithelial cells maintain their structural and functional characteristics in part through their apical tight junctions and adherens junctions. Zonula occludens 1 (ZO-1) is part of the tight junction cytoplasmic plaque that links occludin to the actin cytoskeleton (43). Epithelial adherens junctions are comprised of a cytoskeletal domain, a plaque structure (e.g., β -catenin), and integral membrane components (e.g., E-cadherin). E-cadherin is a transmembrane protein important in calcium-dependent epithelial cell-cell interactions (15).

Alterations in the epithelial junctional complex may be important in the pathogenesis of inflammatory diseases. Disruption of epithelial tight junctions may contribute to leakage of plasma proteins into the airway in asthma (26) and to abnormalities of fluid and electrolyte transport in renal failure (33). Furthermore, expression of a dominant negative cadherin mutant in the small intestine of mice resulted in histopathological features similar to those observed in Crohn's disease (22). It is noteworthy that junctional proteins may be passive targets of inflammatory processes as well as actively influence epithelial inflammatory injury and repair (22, 23).

In inflammatory diseases, leukocytes release cytotoxic compounds during emigration from the vascular space (reviewed in Ref. 25). Recent reports suggest that neutrophil transmigration across endothelium may disrupt adherens junctions by release of proteases (3, 10). However, these observations may be in part due to artifactual release of neutrophil proteolytic activity

Address for reprint requests and other correspondence: G. P. Downey, Clinical Sciences, Rm. 6264 Medical Sciences Bldg., Univ. of Toronto, 1 Kings College Circle, Toronto, Ontario, Canada M5S 1A8 (E-mail: gregory.downey@utoronto.ca).

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during cell permeabilization that is required for immunofluorescence staining (34).

Compared to endothelial transmigration, less is known about leukocyte transmigration across epithelium. In vitro systems suggest that epithelial cells actively facilitate neutrophilic polymorphonuclear leukocyte (PMN) transmigration in the physiological (basolateral to apical) direction (29, 42). A reversible decrease in transepithelial resistance during PMN transmigration may be due to physical disruption of the monolayer or from a PMN-derived secretagogue (37). Recent studies of PMN transmigration have described sites of microinjury in the epithelial monolayer (39). Subsequently, during the repair phase, epithelial cells extend actin-rich lamellipodia into these defects, initiating a process that leads to restitution of the integrity of the epithelial monolayer (39). However, the effects of PMN transmigration on the epithelial apical junctional complex are not known.

The purposes of the present study were 1) to characterize the effects of PMN transmigration on the epithelial adherens junction complex and 2) to determine the mechanism of these effects and their sequelae on epithelial function. The studies reported herein demonstrate that neutrophils migrate by moving primarily between epithelial cells, and this results in transient disruption of interepithelial junctions and later to focal loss of epithelial cells, creating circular defects within the monolayer (microinjury). The disruption of the interepithelial junctions and loss of epithelial cells is in part attributable to leukocyte-derived proteases. These studies provide novel insights into the mechanisms of inflammatory injury to epithelium and suggest potential targets for anti-inflammatory therapy.

MATERIALS AND METHODS

Cell culture and monolayer treatments. Human T84 cells (provided by Dr. P. Sherman, The Hospital for Sick Children, Toronto) were grown in a 1:1 mix of DMEM and Ham's F-12 Nutrient Mix (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Cansera International, Rexdale, Ontario) and 2% (vol/vol) penicillin-streptomycin (GIBCO/BRL) and grown at 37°C in 5% CO₂. T84 cells were either seeded onto inverted polycarbonate filters (1-cm² surface area, 3.0-μm pore, Costar, Cambridge, MA) or grown to confluence in 12-well culture plates. After overnight incubation, filters were placed upright in 12-well culture plates so that the epithelial side was on the lower (dependent) side of the filter. When indicated, epithelial cells were incubated with either 0.5 × 10⁻⁶ M α-1 antitrypsin (Bayer, Toronto, Ontario, Canada, further purified as previously described Ref. 6) for 45 min or EDTA (2 mM for 12 min) to open tight junctions, followed by the addition of 1.5 U/ml of purified leukocyte elastase (Sigma Chemical, St. Louis, MO) for 1 h.

Transepithelial resistance measurements. Transepithelial resistance was measured with a dual-voltage Ohmmeter clamp (World Precision Instruments, Sarasota, FL); readings in ohms were taken by using Ag/AgCl electrodes and were expressed as percentage of baseline. Inserts were used between days 5 and 10 after seeding, when transepithelial electrical resistance was greater than 800 ohms·cm². Mea-

surements were recorded at the start of each experiment and at indicated time intervals.

Neutrophil isolation and transmigration assay. Human PMN (>98% pure) were isolated from citrated whole blood by discontinuous plasma-Percoll gradients (20), resuspended in Hanks' balanced salt solution (HBSS; GIBCO/BRL) and used immediately after isolation. The integrity and nonactivated state of PMN isolated in this manner have been previously validated (12, 20). When indicated, PMN were pretreated with 1) 2.5 mM diisopropylphosphate (DFP), an inhibitor of serine proteases (Calbiochem-Novobiochem, La Jolla, CA) for 2–15 min incubation periods; 2) 12 μM Dupont Merck Pharmaceuticals (DMP)-777, a specific inhibitor of PMN elastase (gift from Dupont Pharmaceutical, Wilmington, DE) for 10 min; 3) 10 μM diphenylene iodonium (DPI, synthesized as previously described) (44) for 2 min; or 4) nitro-L-arginine methyl ester (L-NAME; Sigma Chemical) an inhibitor of nitric oxide synthase. PMN were added to the upper compartment of inserts; transmigration was induced by a chemotactic gradient established with a final concentration of 1 μM *N*-formylmethionyl-leucyl-phenylalanine (fMLP) in the lower compartment. All inserts were washed and incubated in HBSS for 45 min before the addition of PMN.

Morphometric analysis of the epithelial disruption. To quantify epithelial monolayer disruption after induced PMN transmigration, filters were fixed in formyl alcohol and stained with hematoxylin and eosin. The number of defects in the epithelial monolayer was measured at ×100 magnification along the diameter of the filter (1.0 cm), in a transect 1.25 mm wide, comprising 12.5% of the area of the filter. In each filter, the diameters of defects in the epithelium were measured at ×200 magnification; all defects in a vertical transect 0.5 mm in width extending from the periphery of the filter to the filter meridian were measured. If fewer than 20 defects were present in the transect, all defects in repeated transects were measured until the diameters of at least 20 defects in each filter were obtained. The surface area of epithelium lost in each filter was calculated from the diameter of the circular defects and from the density of the defects observed. Epithelial cell diameter was measured by transmitted light microscopy and was found to be 30 μm on average. The measurements were made by an investigator blinded to the treatment protocols of each filter.

Quantification of PMN transmigration. PMN transmigration was measured in two ways. 1) Labeling with calcein AM: PMN were incubated with 1.5 μM calcein AM for 20 min at 37°C and then washed twice. Cells were added to the top chamber of wells, and chemotaxis was induced for 2 h by addition of 1 μM fMLP to the bottom chamber. Fluorescence was measured in the bottom chamber on a fluorescent plate reader (Cytofluor 2300; Millipore, Bedford, MA). The number of cells transmigrated was determined by using standard linear curves of fluorescence vs. known number of PMN separately constructed for each experiment. The fluorescence assay was used for the quantification of all transmigration experiments except those involving the use of DFP. In preliminary experiments, we observed that DFP treatment of PMN interfered with calcein labeling. Therefore, an alternate method (⁵¹Cr labeling) was used for the experiments involving DFP (see below). 2) Labeling with ⁵¹Cr: PMN were incubated with ⁵¹Cr (10 μCi/1 × 10⁶ PMN) for 25 min at 37°C followed by 15 min at 21°C and then washed twice (14). Standard linear curves of counts per minute vs. PMN were constructed. Cells (3 × 10⁶) were added to the top chamber of the wells containing epithelial monolayers, and chemotaxis was induced for 2 h by addition of 1 μM fMLP to the bottom chamber. At the termination of experiments, the number of

cells in each compartment of the chemotaxis chamber was estimated and expressed as a fraction of the total number of PMN retrieved from all compartments of the chamber. At least 75% of the total added radioactivity was recovered in these experiments. Data are from four separate experiments.

Neutrophil chemotaxis assay. PMN, isolated as described above, were labeled with 1.5 μM calcein AM for 20 min at 37°C; 96-well microplate chambers with accompanying 3- μm pore polycarbonate filters (Neuro Probe, Cabin John, MD) were used for this assay. fMLP (1×10^{-7} M, the concentration inducing maximal chemotaxis) was added to the lower compartment, and 25 μl of a suspension containing 4×10^6 PMN/ml was placed on top of the chamber. After a 1-h incubation at 37°C, fluorescence of each well was determined by using a Cytofluor 2300 fluorescent plate reader. The number of PMN that migrated was quantified by using a standard curve (cell number vs. fluorescence) constructed separately for each set of experiments. All assays were done in triplicate, with PMN isolated from at least three separate donors.

Oxidative burst. The reduction of scopoletin was used to measure oxidant production by PMN, as previously described in detail (47). In brief, PMN were incubated in sodium buffer (140 mM NaCl, 4 mM KCl, 10 mM glucose, 10 mM HEPES, 1 mM CaCl₂, pH = 7.4 at 37°C) containing 10.4 μM scopoletin, 9.6 U/ml horseradish peroxidase, 4 mM sodium azide, and either 10^{-7} M phorbol 12-myristate 13-acetate (PMA) or 10^{-7} M fMLP. When indicated, α -1 antitrypsin (0.5×10^{-6} M) was added to wells. Cells were incubated at 37°C for 2 h, and reduction in fluorescence of scopoletin was quantified by use of a fluorescence spectrophotometer. Standard curves were generated by using known amounts of hydrogen peroxide for each experiment.

Flow cytometric analysis of F-actin content. PMN content of F-actin was determined by using the method of Howard and Meyer (24), as previously described (13). In brief, cells were stimulated with either 10^{-7} M PMA or 10^{-8} M fMLP. After stimulation, cells were fixed and permeabilized with a mixture of formalin and lysophosphatidylcholine and then stained with NBD-phalloidin (Molecular Probes, Eugene, OR). Cells were examined on a FACscan flow cytometer (Beckton Dickinson, Palo Alto, CA), and values are expressed as relative fluorescence index.

Intracellular calcium. Intracellular calcium ($[\text{Ca}^{2+}]_i$) was measured by use of fura-2, as previously described (47). In brief, PMN were incubated with 1.5 μM fura 2-AM (Molecular Probes) at 37°C for 30 min, washed, resuspended in HBSS, and transferred to a cuvette. Fluorescence was quantified by using a fluorescence spectrophotometer (Hitachi F-2000) with an excitation wavelength of 346 nm and an emission wavelength of 510 nm. After equilibration, 10^{-7} M fMLP were added, and alterations in fluorescence readings were recorded. Calibration was done by using ionomycin and Mn^{2+} . Data are reported as fold increase above baseline after stimulation with fMLP.

Fixation and immunofluorescence microscopy. Immunofluorescence studies were used to assess the distribution of proteins involved in intercellular adhesion. Initial studies using standard methods of fixation and permeabilization were complicated by an artifactual loss of immunofluorescence of E-cadherin, ZO-1, and β -catenin (16), as has been described for endothelial cells (34). We determined that the optimal conditions for epitope preservation included an initial fixation step with 3% paraformaldehyde for 30 min, followed by incubation with 88% formic acid for 2.5 min before permeabilization with 0.02% Triton X-100 for 2 min. This both preserved epitopes in control slides and prevented postfixation degradation under conditions in which PMN

were present (16). In additional experiments, we determined that methanol fixation (10 min) was optimal for visualization of the actin cytoskeleton. Fixation and permeabilization were done at 21°C, and samples were rinsed three times in PBS between all steps before blocking with 1% bovine serum albumin in PBS (10 min).

Primary antibodies [monoclonal antibodies against E-cadherin, β -catenin; polyclonal antibody against ZO-1 (Zymed Laboratories, San Francisco, CA)] were added at 1:100 dilution, and monolayers were incubated for 45 min at 37°C. After washing, cells were incubated for 45 min at 37°C with FITC, trimethylrhodamine isothiocyanate, or indocarbocyanine-conjugated secondary antibody (Jackson Laboratories, West Grove, PA) at a dilution of 1:100. For most experiments involving double labeling, fixed cells were coincubated with both primary antibodies (as indicated) before incubation with secondary antibodies. However, in experiments in which biotinylated anti-CD15 (Sigma Chemical) was used to label PMN, the monolayers were initially incubated with primary then secondary antibody against intercellular junctional proteins and then kept overnight at 4°C. The following day, biotin-conjugated anti-CD15 antibody (Sigma Chemical) was added at 1:100 dilution for 45 min at 37°C. Fixed cells were then washed and incubated for 30 min at 37°C with a 1:20 dilution of FITC-streptavidin. For double-labeling experiments involving visualization of the actin cytoskeleton, cells were coincubated with the appropriate primary antibody (as above) and trimethylrhodamine isothiocyanate-phalloidin at 1:100 dilution for 45 min at 37°C. Fixed cells were then washed and incubated for 45 min at 37°C with the appropriate secondary antibody at a dilution of 1:100. After two additional washes, monolayers were mounted with fluorescent mounting media (DAKO, Carpinteria, CA) onto slides and examined by confocal fluorescence microscopy.

SDS-PAGE and immunoblotting. For SDS-PAGE of whole cell extracts, filter inserts were excised and placed directly into boiling lysis buffer (2% SDS, 10% glycerol, 65 mM Tris/HCl, pH 6.8, 50 mM dithiothreitol) (27). For SDS-PAGE analysis of the supernatant from the apical surface of the monolayer, samples from the lower chamber of eight wells (total 8 ml) were pooled, protease inhibitors were added (1 mM PMSF, 0.5 mM benzamide, 10 $\mu\text{g/ml}$ aprotinin, and 10 $\mu\text{g/ml}$ leupeptin), and samples were centrifuged (5 min at 900 rpm). Supernatant was concentrated to 1 ml by using Centriprep (Centrifugal Filter Devices) with a molecular mass exclusion of <10 kDa (Millipore, Bedford, MA). After electrophoresis, proteins were transferred to nitrocellulose membranes, blocked with 5% low fat milk in PBS with 0.05% Tween-20, and incubated overnight at 4°C with the relevant antibodies. The primary antibodies were those used for immunofluorescence studies with the exceptions of anti-E-cadherin (Sigma Chemical), anti- β -catenin (Transduction Laboratories, Lexington, KY), and cytokeratin 18 (Serotec, Raleigh, NC). After washing, blots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG at 1:5,000 dilution for 1 h at room temperature and developed by use of an enhanced chemiluminescence system according to manufacturer's instructions (Amersham Canada, Oakville, ON). When indicated, blots were reprobed with cytokeratin 18 to correct for small differences in protein loading. Quantitative densitometric analysis of the blots was done by using commercial image analysis software (IP Lab Gel), and the intensity of all blots was within the linear range of the film.

Protease inhibition. Several strategies were used to inactivate leukocyte proteases. To inhibit cell-associated proteases, including elastase, PMN were pretreated either with

DFP, a serine protease inhibitor, or DMP-777, a specific neutrophil elastase inhibitor. Alternately, extracellular proteases were inactivated by addition of α -1-antitrypsin ($8 \text{ nmol/l} \times 10^6 \text{ PMN}$) to buffer. In experiments conducted to measure extracellular release of elastase, PMN (10×10^6) were preincubated with protease inhibitors or vehicle control for the indicated times before stimulation with 10^{-6} M fMLP in the presence of cytochalasin B ($5 \mu\text{M}$). After 1 h, cells were centrifuged, and supernatant was immediately frozen.

Elastase assay. Neutrophil elastase activity was determined on samples from apical supernatant of the epithelial cultures after either maximal stimulation of PMN primary granule release or PMN transepithelial migration. To measure maximal primary granule release, the chromogenic substrate specific to human neutrophil elastase, methoxysuccinyl alanyl alanyl prolyl valyl *p*-nitroanilide (MeOSAAPVpNA, Sigma Chemical) (7) was used. Purified human neutrophil elastase (Elastin Products, Owensville, MO) was used to establish a standard reference. Samples were diluted 1:10 in 100 mM HEPES, 0.5 M NaCl, and 0.1% Brij 35 at pH 7.5 and reacted with 1 mM MeOSAAPVpNA. The change in absorbance per minute at a wavelength of 410 nm was recorded in a spectrophotometer (model DU7, Beckman Instruments). Sample elastase concentrations were determined by referring to the standard reference curve. To measure elastase activity in supernatant after PMN transepithelial migration, the EnzChek Elastase Assay Kit (Molecular Probes) was used, as per the manufacturer's instructions.

Myeloperoxidase assay. Neutrophil myeloperoxidase activity was determined in thawed samples from supernatant after PMN transepithelial migration. Supernatant was added to assay buffer containing Na_2PO_4 , 0.3% H_2O_2 , and 0.1% *o*-dianisidine hydrochloride. The H_2O_2 -dependent oxidation of *o*-dianisidine hydrochloride was used as an index of myeloperoxidase activity. One unit of myeloperoxidase was defined as the amount of myeloperoxidase required to degrade $1 \mu\text{mol H}_2\text{O}_2/\text{min}$ at 25°C . Myeloperoxidase values were compared between samples of equal volume of supernatant.

Statistical analysis. Parametric data were compared by using *t*-tests for mean values or analysis of variance with correction for multiple comparisons (Scheffé's test) when more than two variables were analyzed.

RESULTS

Neutrophil transmigration and epithelial monolayer disruption. PMN were induced to transmigrate across monolayers of T84 epithelial cells in the physiological basolateral-apical direction by a transepithelial gradient of fMLP, a potent PMN chemoattractant. To study structural alterations induced by PMN transmigration, we examined the monolayers microscopically. Single PMN infiltrated the monolayer as early as 20–30 min (Fig. 1, *B* and *C*), but at later time points (1–2 h), PMN moved through the monolayer in clusters associated with disruption of the monolayer (Fig. 1, *D* and *E*). The formation of circular defects (holes) in the monolayer coincided with the appearance of free epithelial cells in the apical culture media, indicating that neutrophil transmigration led to epithelial detachment. By 18 h, the majority of PMN had traversed the epithelial monolayer and subsequently detached from the apical surface, leaving multiple circular defects in the monolayer (Fig. 1*E*). The epithelial cells remaining

attached to the membrane were physiologically viable as determined by their ability to exclude both trypan blue and propidium iodide (data not shown), indicating that the effects of the neutrophils on the epithelium were spatially delimited.

When the number of PMN added to the basolateral surface of the monolayer was increased, the number of PMN that transmigrated completely across the monolayer and the number and size of defects in the epithelium increased proportionately (Fig. 2). The addition of twice as many PMN ($1.5 \text{ vs. } 3.0 \times 10^6$) to the basal surface resulted in a proportionate increase in loss of epithelial cells from the monolayer at 2 h ($6 \times 10^3 \text{ vs. } 1 \times 10^4$ epithelial cells lost/ cm^2 , respectively). By 18 h, although the number of defects decreased, the size of the remaining defects increased, resulting in a greater net loss of epithelial surface area (Fig. 2).

Leukocyte-derived oxidants are not responsible for alterations in epithelial apical junctions. To traverse the epithelial monolayer, PMN must somehow induce disruption of epithelial attachments either to the substratum or to other epithelial cells (intercellular junctions). PMN possess a number of potent antimicrobial systems that could effect these alterations in the epithelial alterations during transmigration. For example, oxidants generated by either the NADPH oxidase (O_2^- , H_2O_2 , HOCl) or nitric oxide synthase ($\text{NO}\cdot$, ONOO) could be envisioned to lead to epithelial disruption. To investigate this possibility, PMN were pretreated with either DPI, an inhibitor of the NADPH oxidase (17, 40), or L-NAME, an inhibitor of nitric oxide synthase (31). However, neither DPI nor L-NAME pretreatment attenuated the fall in resistance associated with transmigration (data not shown) nor decreased transepithelial PMN migration when compared with untreated PMN (DPI: $1.32 \pm 0.25 \times 10^6$ cells; L-NAME: $0.82 \pm 0.12 \times 10^6$ cells; untreated: $1.04 \pm 0.11 \times 10^6$ cells per 3×10^6 cells added, $P > 0.25$). These observations suggest that PMN-derived oxidants are not involved in PMN transmigration through monolayers, in accordance with the observations of others (38).

Neutrophil proteases facilitate transepithelial migration. In addition to oxidants, leukocytes can release proteases such as elastase that could mediate proteolytic cleavage of the epithelial attachments (either cell-cell or cell-substratum) and thus facilitate transmigration of PMN through the epithelial monolayer. If this concept is correct, the prediction is that protease inhibitors should interfere with PMN transmigration across the epithelium. This possibility was investigated by using several chemically distinct protease inhibitors. The high molecular weight protease inhibitor α -1-antitrypsin was used to inhibit proteases released into the extracellular space. Alternately, PMN were pretreated with one of two cell-permeant protease inhibitors, DFP (an irreversible serine protease inhibitor; Refs. 28, 30) or DMP-777 (a specific elastase inhibitor; Refs. 9, 35, 46). Pretreatment of cells with either DFP or DMP-777 would be expected to neutralize proteases such as elastase contained within neutrophil granules and thus completely abrogate their effects during

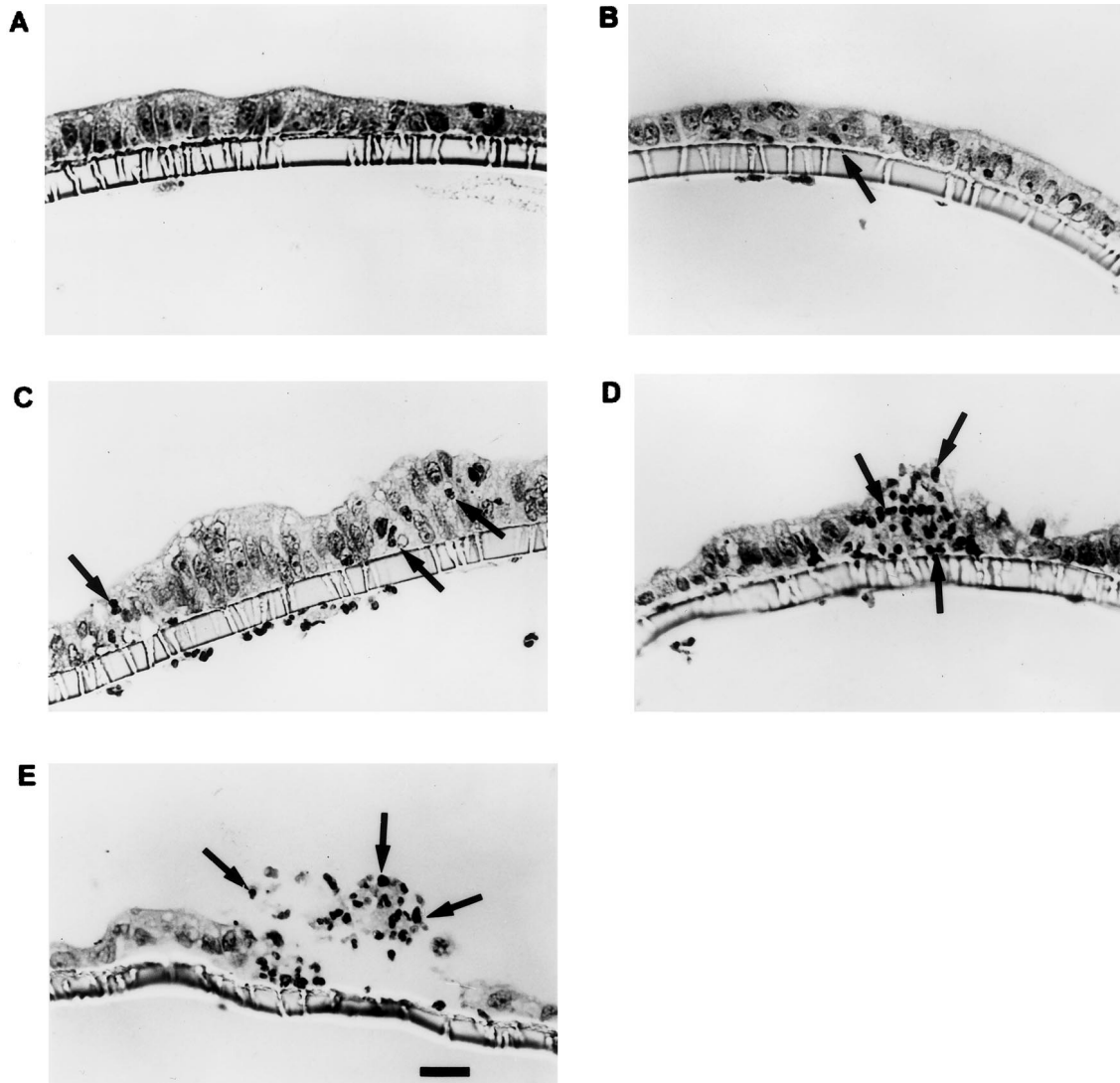


Fig. 1. Neutrophilic polymorphonuclear leukocytes (PMN) transmigrate singly and then in clusters across epithelium. Epithelial cells form confluent monolayers when grown on filter supports (A). After induction of PMN transepithelial migration, PMN initially transmigrate singly across monolayers (B: 30 min and C: 45 min after PMN addition) and then form clusters within the epithelium (D: 90 min after PMN addition). Finally, PMN transmigration results in structural disruption of the monolayer with focal detachment of epithelial cells (E: 120 min after PMN addition). Filters were fixed in formyl alcohol, stained with hematoxylin and eosin, embedded in paraffin, and sectioned. Arrows indicate PMN. Bar represents 10 μm. Magnification ×400.

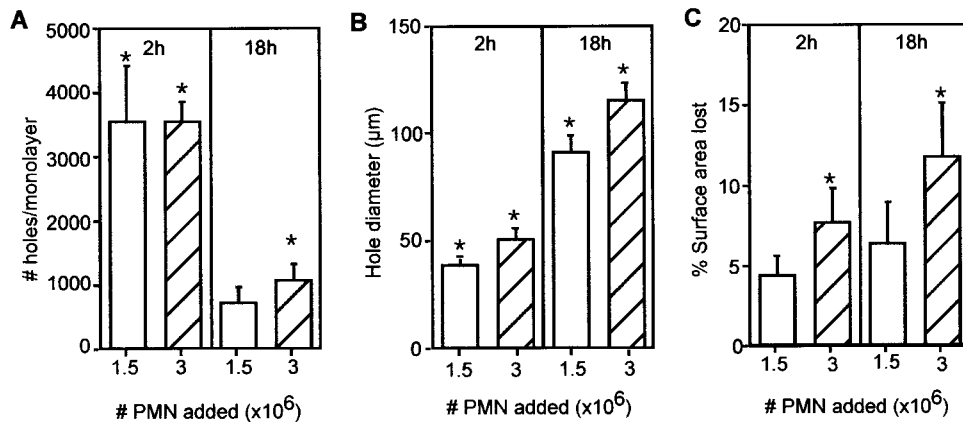


Fig. 2. PMN transmigration disrupts normal epithelial morphology. PMN (1.5 or 3 million) were added to the basal surface of monolayers and induced to transmigrate across a chemotactic gradient *N*-formylmethionyl-leucyl-phenyl-alanine (fMLP; 10⁻⁶ M) for 2 h. Number of PMN that completed transmigration was 0.42 ± 0.14 × 10⁶ and 1.43 ± 0.28 × 10⁶ for 1.5 and 3 million PMN added, respectively. Data for no. of holes per monolayer (A), hole diameter (B), and %surface area lost (C) are means ± SE from 3 separate experiments. *Values differ significantly from control (monolayer without PMN; no defects detected), *P* < 0.05 (ANOVA, corrected with Scheffé's test).

transmigration. To ensure that the agents used actually inhibited PMN elastase under the conditions of our experiments, we quantified elastase activity after maximal stimulation of PMN azurophilic granule release. Incubation of PMN with α -1-antitrypsin resulted in partial inhibition whereas pretreatment of PMN with either DFP or DMP-777 resulted in complete inhibition of extracellular elastase activity (Table 1).

Having established the effectiveness of the elastase inhibitors, we next sought to define the role of leukocyte-derived proteases in transepithelial migration. First, epithelial monolayers were pretreated with the high molecular weight anti-protease α -1-antitrypsin before PMN transmigration, and its presence was maintained throughout the experiment (8). Under these conditions, PMN transmigration across epithelial monolayers was decreased fivefold, compared with untreated monolayers (Table 2). In contrast, PMN chemotaxis across identical filters in the absence of an epithelial monolayer was unaffected by α -1-antitrypsin (data not shown), indicating that inhibition of proteases in the extracellular space specifically affected the ability of PMN to transmigrate across epithelium without diminishing their generalized chemotactic responsiveness. Pretreatment of epithelial monolayers with α -1-antitrypsin also significantly reduced the degree of PMN-associated structural disruption of the monolayer; fewer defects were associated with PMN transmigration in the presence of this protease inhibitor than in its absence. Compared with untreated epithelium, loss of epithelial surface area was decreased in the presence of α -1-antitrypsin as indicated by several parameters including the surface area lost ($0.45 \pm 0.05\%$; $P < 0.05$), average diameter of the defects ($18.5 \pm 0.5 \mu\text{m}$; $P < 0.05$), and the number of defects (175 ± 42 ; $P < 0.05$).

Treatment of PMN with α -1-antitrypsin did not, however, result in a nonspecific decrease in cell responsiveness. For example, fMLP-induced increases in $[\text{Ca}^{2+}]_i$ [control 4.9 ± 1.0 - vs. treated 4.5 ± 1.1 -fold increase (mean \pm SD $n = 3$) above baseline], oxidant production, and actin assembly (data not shown) were not affected by α -1-antitrypsin.

The effect of the cell-permeant protease inhibitors on PMN transmigration across the epithelial monolayer was studied next. For these experiments, PMN were

Table 1. PMN elastase activity

PMN	Elastase Activity, mM
Untreated	578 \pm 48
α -1-Antitrypsin	325 \pm 60*
DFP	0 \pm 0*
DMP-777	0 \pm 0*

Values are means \pm SD. Elastase activity was measured in the supernatant after maximal stimulation of 10×10^6 neutrophilic polymorphonuclear leukocyte (PMN) primary granule release using *N*-formylmethionyl-leucyl-phenylalanine (fMLP) after treatment with cytochalasin B. DFP, diisopropylphosphate; DMP-777, Dupont Merck Pharmaceuticals-777. * $P < 0.05$ by ANOVA

Table 2. PMN migration across epithelial monolayers

PMN Treatment	% PMN Transmigrated
Control†	54 \pm 4
DFP†	12 \pm 4*
Control‡	27 \pm 6
α -1-Antitrypsin‡	5 \pm 1*
DMP-777‡	3 \pm 2*

Values are means \pm SD. PMN were labeled with ^{51}Cr or calcein-AM, added to the basal aspect of monolayers grown on filters and induced to transmigrate for 2 h (fMLP 10^{-6} M). *Values differ significantly from control, $P < 0.05$ by ANOVA.

labeled with either ^{51}Cr or calcein AM to allow quantitative analysis of transmigration. When PMN were pretreated with DFP (a nonspecific serine protease inhibitor) and induced to transmigrate across epithelial monolayers with a transepithelial gradient of fMLP, PMN transmigration was reduced more than fourfold (Table 2). PMN pretreatment with DMP-777, a specific elastase inhibitor, resulted in ninefold fewer treated PMN crossing the monolayer compared with control (Table 2). These studies indicate that the activity of serine proteases such as elastase facilitates PMN transepithelial migration.

To study the role of leukocyte-derived proteases in alterations in the epithelial barrier function resulting from PMN transmigration, the effects of the protease inhibitors on PMN-induced alterations in transepithelial resistance were monitored. PMN transmigration resulted in a fall in transepithelial resistance that was proportional to the number of cells transmigrating. Pretreatment of PMN with either DFP or DMP-777 significantly attenuated the fall of transepithelial resistance during transmigration when compared with untreated cells (Fig. 3).

The observed decrease in PMN transmigration and the attenuation of the fall in transepithelial resistance by protease inhibitors might be accounted for by several factors, including a generalized inhibition of PMN chemotactic ability, prevention of epithelial disruption by PMN-derived products, or a combination thereof. To assess the contribution of each of these factors, additional studies were undertaken to study the effects of these inhibitors on the chemotactic function of PMN using micro-Boyden chambers. These studies demonstrated that, in the absence of epithelial cells, neither DFP nor DMP-777 treatment significantly inhibited the chemotactic response of PMN to fMLP (Table 3). Additionally, neither DFP nor DMP-777 treatment affected fMLP-induced increases in $[\text{Ca}^{2+}]_i$ [control 4.3 ± 0.6 vs. DFP 3.3 ± 0.8 vs. DMP 3.2 ± 0.9 -fold increase (mean \pm SD; $n = 3$) above baseline]. Taken together, these data indicate that these protease inhibitors do not induce a generalized state of hyporesponsiveness of PMN but rather selectively interfere with the processes involved in transepithelial migration.

Neutrophil transmigration is associated with alterations in the epithelial junctional complex. Our studies suggested that PMN transmigration resulted in disruption of the epithelial monolayer with detachment

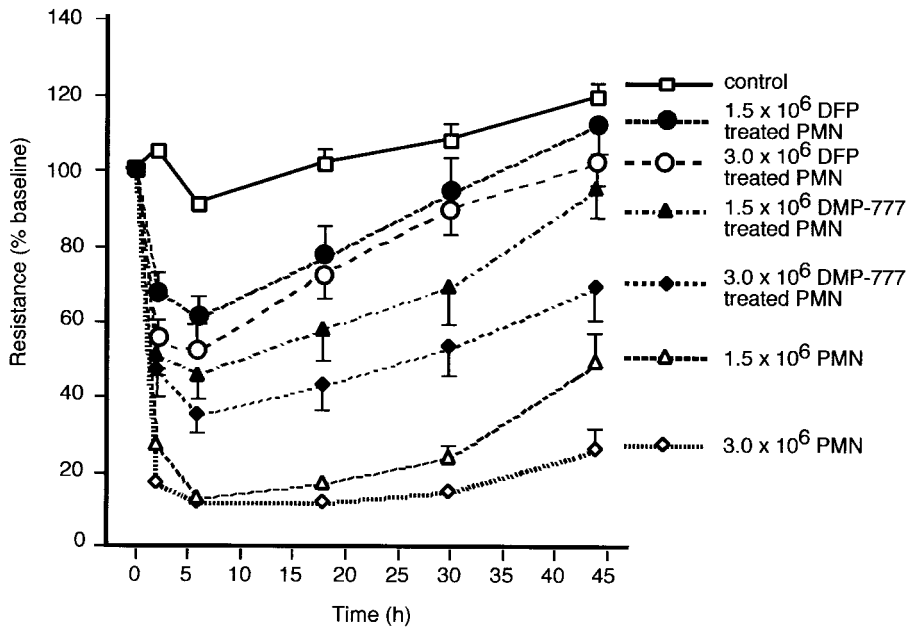


Fig. 3. PMN transmigration across epithelial monolayers results in loss of transepithelial resistance (TER). PMN transmigration across monolayers of T84 epithelial cells was induced using fMLP (10^{-6} M) for 2 h. In control monolayers, TER gradually increased over time with small changes related to changes of the media. After addition of PMN, TER fell rapidly within the first 2 h of PMN transmigration. Recovery toward baseline was noted after 18 h and was more rapid and complete in monolayers where fewer PMN were initially added. Diisopropylphosphate (DFP) pretreatment of PMN significantly decreased the magnitude of TER loss, permitting complete recovery of TER to baseline by 45 h (ANOVA $P < 0.01$ for each time point). Similarly, pretreatment of PMN with Dupont-Merck Pharmaceuticals (DMP)-777, a specific inhibitor of elastase, significantly attenuated the fall in resistance associated with PMN transmigration. Data are means \pm SE from a minimum of 4 separate experiments.

and loss of epithelial cells. To examine the morphological alterations in the monolayer that resulted from PMN transmigration in more detail, we utilized confocal immunofluorescence microscopy. In agreement with transmitted light microscopic studies, focal areas of epithelial disruption were observed by immunofluorescence microscopy using rhodamine phalloidin to label the actin cytoskeleton of epithelial cells and anti-CD15 to label the PMN (data not shown).

Focal loss of immunofluorescence staining of junctional proteins was observed during PMN transmigration through the monolayer. In particular, the adherens junction proteins E-cadherin and β -catenin of the epithelial cell bordering the sites of PMN infiltration demonstrated localized disruption and/or dissolution (Fig. 4, C and E). ZO-1, a tight junction protein, was also focally disrupted, but the extent of this was less than for E-cadherin and β -catenin (Fig. 4, D and F). Such disruption of epithelial cell-cell interactions may be integral to PMN movement through an epithelial monolayer by allowing PMN to pass between epithelial cells.

To quantify this apparent loss of E-cadherin, the immunofluorescence intensity of cell borders (measured as mean pixel intensity) immediately adjacent to infiltrating PMN was compared with the contralateral

border of the same cell. With the use of this strategy, E-cadherin fluorescence adjacent to the sites of PMN infiltration was significantly diminished compared with that at the opposing border of the same cell (mean fluorescence intensity 57 ± 8 vs. 107 ± 5 , respectively; $n = 4$, $P < 0.01$). ZO-1 fluorescence intensity, however, was similar in both regions (average intensity 89 ± 7 vs. 93 ± 6 , respectively, $P = 0.7$).

Late alterations in apical junctional proteins. Recent studies have suggested that apical junction proteins modulate epithelial migration and monolayer formation during repair of epithelial wounds (1). We endeavored to characterize alterations in epithelial apical junctional proteins that occurred subsequent to PMN infiltration. Our goal was to determine whether the loss and/or redistribution of these proteins observed during the early phases of PMN infiltration persisted at later time points and during repair of the epithelium. To define the distribution of intercellular proteins at the sites of epithelial loss (microinjury), we studied epithelial intercellular junctions 18 and 30 h after the addition of PMN. After fixation, double labeling with rhodamine phalloidin to visualize the actin cytoskeleton of the epithelium (data not shown) and antibodies to either E-cadherin or β -catenin to visualize the junctional proteins revealed circular defects in the epithelium associated with focal loss of E-cadherin (Fig. 4G) and β -catenin immunofluorescence (data not shown). To evaluate relative alterations in adherens junction and tight junction protein distribution, double-label immunofluorescence with combinations of either anti-E-cadherin and anti-ZO-1 or anti- β -catenin and anti-ZO-1 antibodies was utilized. These studies revealed that E-cadherin (Fig. 4G) and β -catenin (not shown) immunofluorescence signals were decreased at the epithelial cell membrane bordering these regions, whereas staining of ZO-1, a tight junction protein well

Table 3. Neutrophil chemotaxis assay

PMN Treatment	No. of PMN Migrating
Control	81,095 \pm 8,163
DFP	65,312 \pm 8,818
DMP-777	86,297 \pm 7,091

Values are means \pm SD. Microchemotaxis chamber assay; data are presented for fMLP concentration (10^{-8} M) inducing maximal chemotaxis. 100,000 cells added/filter, data from 3 separate PMN donors, conditions for each donor done in triplicate. $P > 0.15$ by ANOVA.

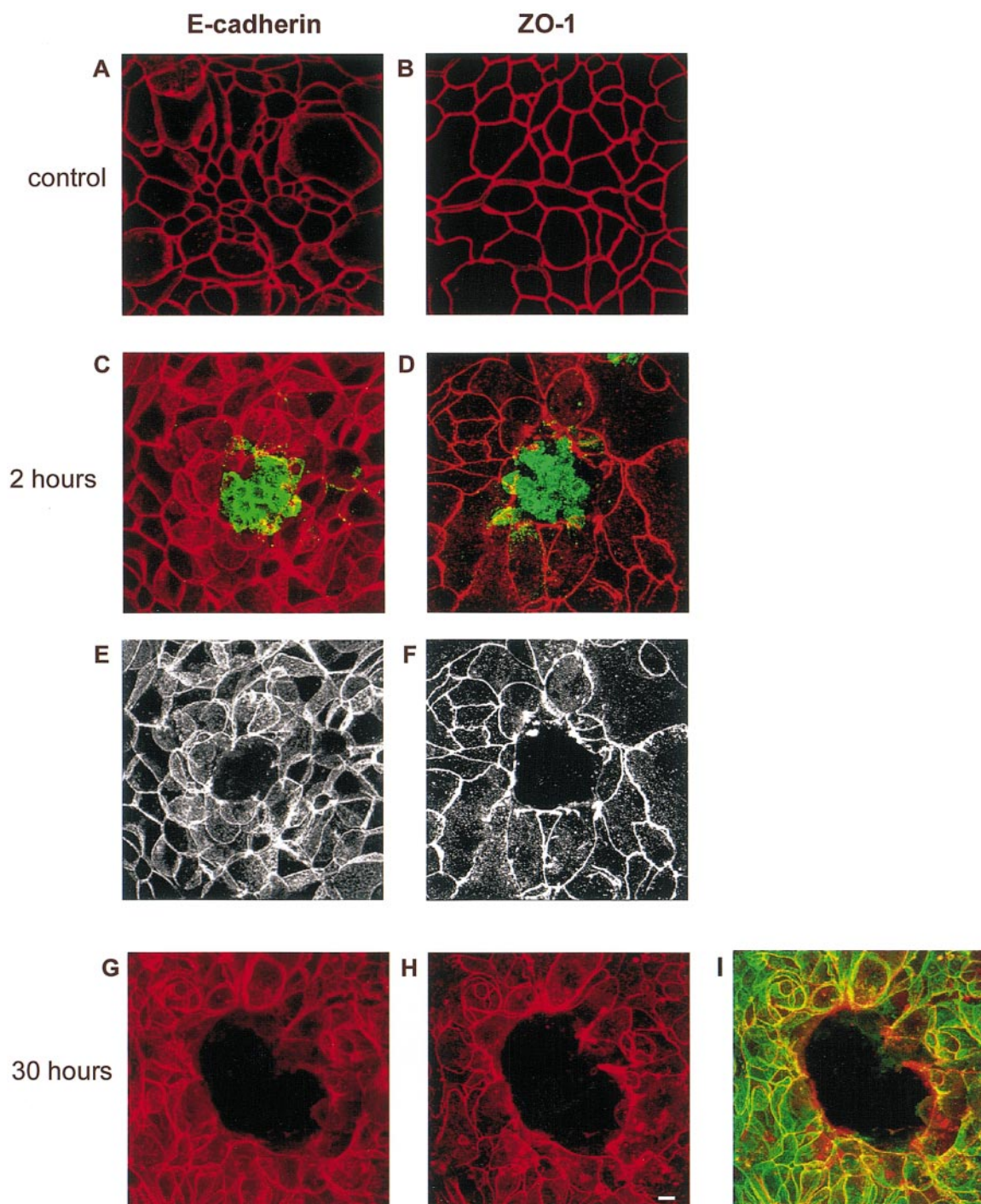


Fig. 4. Loss of junctional proteins immunofluorescence during PMN transmigration through epithelial monolayers. After 2 h of induced PMN transmigration, epithelial monolayers were either fixed and labeled immediately or washed and incubated in media for an additional 28 h. PMN were labeled with anti-CD15 (green in *C* and *D*). Resting monolayers demonstrated uniform distribution of E-cadherin (*A*) and zonula occludens 1 (ZO-1; *B*). At 2 h, PMN infiltration of monolayers was associated with focal loss of E-cadherin (red in *C* and white in *E*, same images) and ZO-1 (red in *D* and white in *F*, same images). E-cadherin (*C* and *E*) and ZO-1 (*D* and *F*) were diminished at the margin of epithelial cells adjacent to the PMN cluster (*C* and *E*). Monolayers fixed 30 h after induced PMN transmigration showed loss of epithelial cells from sites of PMN-associated defects. E-cadherin (*G*), in contrast to ZO-1 (*H*, same image as *G*), was decreased at cell borders immediately adjacent to defects. *I*: merged image in which E-cadherin is seen in green and ZO-1 is red. Data for β -catenin (not shown) were similar to E-cadherin. Images were acquired by confocal microscopy ($\times 63$ magnification). Each *X-Y* image is an overlay of 30–40 sections $0.5 \mu\text{m}$ apart to represent accurately protein distribution throughout the monolayer. Filters were fixed using the modified fixation protocol described in METHODS. Bar represents $10 \mu\text{m}$.

localized to the apical epithelial surface, was relatively preserved (Fig. 4, *H* and *I*).

To quantify alterations in the distribution of adherens junction proteins in proximity to sites of defects during the phase of epithelial repair, we calculated the ratio of E-cadherin and β -catenin to ZO-1 fluorescence. At both 18 and 30 h, the ratios of E-cadherin and β -catenin to ZO-1 were diminished at the perimeter of sites of microinjury compared with regions in the same field where the epithelial monolayer was intact (Fig. 5). Furthermore, the absolute values of E-cadherin and β -catenin immunofluorescence were significantly lower at the sites of microinjury than in control regions (data not shown), indicating that the decreased ratio of adherens junction proteins to ZO-1 represented an absolute decrease in adherens junction proteins and not simply a change in their relative abundance.

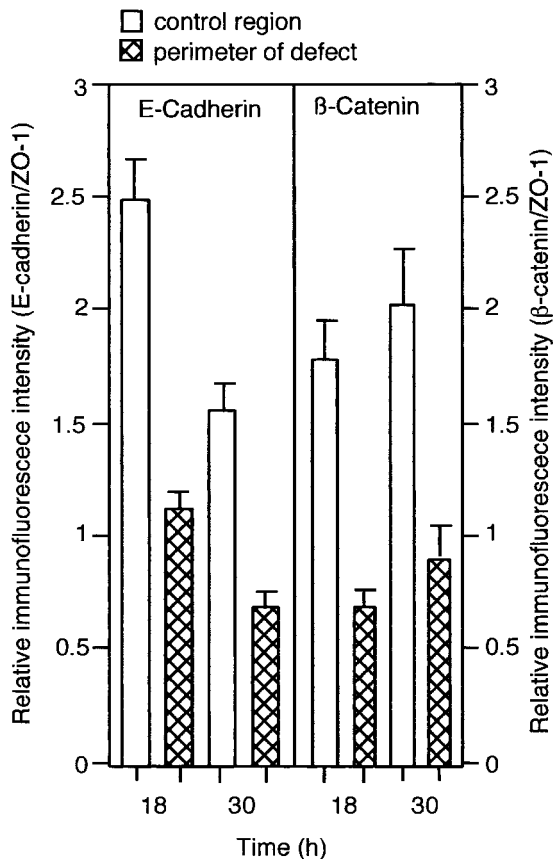


Fig. 5. Selective loss of adherens junction protein immunofluorescence is associated with PMN transmigration. After 2 h of induced PMN transmigration, epithelial monolayers were washed in Hanks' balanced salt solution and incubated in media for an additional 18 or 30 h. Filters were then fixed in paraformaldehyde, treated with formic acid, and permeabilized. Double labeling was done with either anti-E-cadherin and ZO-1 or anti- β -catenin and ZO-1. Ratios of E-cadherin or β -catenin to ZO-1 immunofluorescence intensity at cell borders immediately adjacent to the PMN induced monolayer defects were compared with areas of the image in which the monolayer was intact. The relative immunofluorescence intensity was significantly lower for both E-cadherin and β -catenin at 18 and 30 h ($P < 0.01$). Absolute levels of ZO-1 were not significantly different in the two regions. Images were collected by confocal microscopy with an extended depth of 20 μ m, 40 overlaid sections. Images are representative of 10 different sections.

Evidence of limited proteolytic degradation of adherens junction proteins. To determine the contribution of proteolytic degradation to the loss of adherens junction protein immunofluorescence staining at sites of microinjury, we initially utilized immunoblotting to quantify the amount of adherens junction and other proteins in the epithelial monolayers. Cells from the entire filter insert were lysed directly in identical volumes of denaturing lysis buffer and subjected to SDS-PAGE and immunoblot analysis. E-cadherin and β -catenin levels were normalized to that of cytokeratin 18 (Fig. 6A, top). No significant change in E-cadherin ($88 \pm 11\%$) or β -catenin levels ($101 \pm 9\%$) after 2 h of induced PMN transmigration was observed. Thus, within the limits of sensitivity of Western blotting, degradation of adherens junction proteins at 2 h after induced PMN transmigration could not be detected. Similarly, no decrease in the tight junction protein, ZO-1, was observed (data not shown).

We considered the possibility that, even in the absence of gross changes, PMN transmigration might result in limited degradation of junctional proteins. This activity could result in release of proteolytic fragments into the apical supernatant. To investigate this, we collected and concentrated the supernatant from the apical surface of the epithelial monolayer after 2 h of induced PMN transepithelial migration. Using an antibody known to recognize an epitope from the extracellular domain of E-cadherin, we detected degradation products of E-cadherin in the supernatant (Fig. 6A, bottom). These observations indicate that there is limited proteolytic degradation of junctional proteins during PMN transmigration with release of degradation products of E-cadherin into the supernatant.

To determine the role of PMN proteases in the degradation of E-cadherin observed during PMN transepithelial migration, several experiments were conducted. First, Western analysis of the supernatant from the lower chamber (apical side of monolayer) was conducted in the presence and absence of protease inhibitors. As illustrated in Fig. 6B, E-cadherin degradation products were not detected in the apical supernatant when PMN were pretreated with DMP-777 (a cell permeant specific inhibitor of leukocyte elastase). Second, the supernatant from the lower chamber was assayed for elastase and myeloperoxidase (markers of primary granule secretion). These experiments revealed that the levels of both enzymes were significantly increased in wells after fMLP-induced PMN transepithelial migration (52.0 ± 10.6 ng elastase/well and 73.9 ± 17.6 μ g/well myeloperoxidase). Third, purified leukocyte elastase was added to the apical supernatant of confluent monolayers of epithelial cells. Initial studies, however, determined that this treatment did not result in release of E-cadherin degradation products (Fig. 6C, lane 3). We reasoned that when the monolayer was intact, elastase added to the apical surface might not have access to the more basally situated junctional proteins. In an attempt to permit access of elastase to the epithelial basolateral space, EDTA was then added to disrupt the tight junctions

before addition of elastase. Under these conditions, E-cadherin degradation fragments including one of 23 kDa were released into the supernatant of the apical surface of the epithelial cells and detected by an antibody against the extracellular portion of E-cadherin (Fig. 6C, lane 4). However, consistent with the results obtained by Western analysis of epithelial monolayers after PMN transmigration, there was no significant change in total E-cadherin levels after the addition of purified elastase to monolayers either in the presence ($83 \pm 8\%$ control) or absence ($100 \pm 19\%$ control) of EDTA. As previously described, for these experiments,

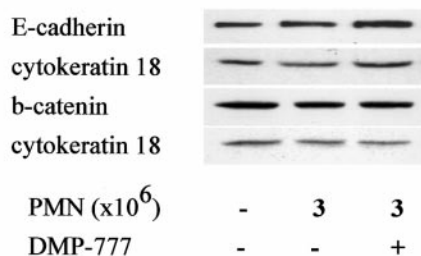
E-cadherin levels were normalized to cytokeratin 18 levels to ensure equal loading of epithelial proteins (Fig. 6D).

DISCUSSION

PMN transmigration across epithelium in many inflammatory conditions is associated with injury to and dysfunction of the epithelial barrier. Our data demonstrate that PMN transmigration leads to loss of epithelial cells from the monolayer, creating defects (areas of microinjury) that are spatially associated with clustered PMN and a localized disruption of E-cadherin. Release of proteolytic fragments of E-cadherin into the supernatant occurred during transmigration and was prevented by elastase inhibitors, consistent with spatially delimited degradation of this adherens junction protein by leukocyte-derived proteases. The notion that leukocyte-derived elastase is important in the pathogenesis of epithelial injury in inflammatory bowel disease is supported by animal models of colitis in which the administration of anti-elastase compounds ameliorates intestinal damage (36) and by reports that levels of elastase are elevated in the gut lumen of patients with inflammatory bowel disease (2, 19).

After the initial phase of PMN transmigration, during which individual ("scout") PMN passed between epithelial cells, PMN did not advance randomly but rather traversed the monolayer in clusters associated with areas of epithelial loss. Sites in the monolayer

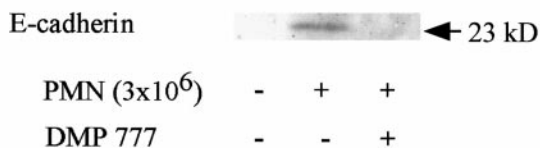
A Cell Monolayer



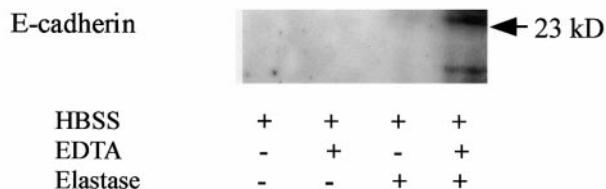
Supernatant



B Supernatant



C Supernatant



D Cell monolayer



Fig. 6. Adherens junction proteins are partly degraded during PMN transepithelial migration. **A:** in cell monolayer, PMN transmigration was induced for 2 h, after which protein was extracted and analyzed by SDS-PAGE and Western blotting with anti-E-cadherin and anti- β -catenin antibodies. No difference in protein concentration was detected between control monolayers and those subject to PMN transmigration. Blots were stripped and reprobed with anticytokeratin 18 to normalize for amount of protein loaded in all lanes by densitometric analysis; the band representing cytokeratin 18 immunoreactivity is shown for the respective E-cadherin and β -catenin blots above. Representative blots ($n = 3$ different experiments). In supernatant, after 2 h of PMN transmigration, buffer from the lower Transwell chamber was collected, concentrated, and analyzed by SDS-PAGE and Western blotting with anti-E-cadherin antibody. Degradation products of E-cadherin were detected in the buffer (shown on blot), but not monolayer, portion. *Lane 1*, no PMN added; *lanes 2* and *3*, PMN from 2 different donors. **B:** PMN were either untreated or pretreated with DMP-777 and induced to transmigrate through epithelium, as indicated. After 2 h of induced transmigration, buffer from the lower Transwell chamber was collected and analyzed for degradation products of E-cadherin, as above. Epithelial cells, grown to confluence, were either treated with buffer control or EDTA to open tight junctions and then incubated for 1 h with purified leukocyte elastase. **C:** supernatant was collected and analyzed by SDS-PAGE and Western blotting with anti-E-cadherin antibody. Degradation products of E-cadherin were detected in the buffer after elastase treatment. **D:** protein was extracted from the monolayers and analyzed by SDS-PAGE and Western blotting with anti-E-cadherin antibody. No difference in protein concentration was detected between control monolayers and those treated with elastase. Blots were stripped and reprobed with anticytokeratin 18 to normalize amounts of protein loading for densitometry; the band representing cytokeratin 18 immunoreactivity is shown for the respective E-cadherin blot above. Representative blots ($n = 3$ different experiments).

through which the initial PMN pass may represent areas of weakness in intercellular junctions. Passage of scout PMN through these areas might further promote additional PMN transmigration by several mechanisms, such as disruption of intercellular junctions resulting in microdomains of an increased chemotactic gradient. Quantitative analysis supported the concept that PMN traversed the monolayer primarily in groups and migrated preferentially through areas of epithelial loss. Specifically, doubling the number of PMN added to the basal surface resulted in a threefold increase in the number of PMN that completely transmigrated the monolayer, whereas the loss of epithelial surface area only increased by a factor of 1.7. Thus, when increased numbers PMN were added to the basal epithelial surface, relatively more PMN transmigrated per unit of epithelial surface area lost (9.54×10^3 vs. 18.6×10^3 PMN transmigrated/mm² of surface area lost, respectively).

Disrupted E-cadherin staining at sites of PMN infiltration could be accounted for by several different mechanisms. PMN may physically displace epithelial junctional proteins. Alternatively, the junctional proteins may redistribute away from the site of PMN infiltration, either remaining on the cell surface or becoming internalized. In the present study, we provide evidence for a third possibility, specifically that there is limited proteolytic degradation of E-cadherin mediated by neutrophil elastase.

It is noteworthy that we observed a diminution of E-cadherin immunofluorescence at the periphery of migrating epithelial cells during the later phase of repair of the monolayer. Although morphologically similar, the changes in the epithelial apical junctional proteins during the initial phase of PMN transmigration and later during monolayer repair may be mechanistically distinct. Various mechanisms have been proposed that facilitate epithelial wound closure in other model systems. Early experiments conducted with adult epidermal epithelial cells demonstrated that lamellipodia extend into wounds during epithelial repair (32). In embryonic epidermis, an actin cable was visualized at the edge of wounds and postulated to act as a contractile "purse string" during wound closure (32). Similarly, experiments using cell lines suggest that both lamellipodial extension and formation of actin filament rings (5, 39) occur during epithelial repair processes.

The potential role of E-cadherin in epithelial cell migration is complex. Reduction in adherens junction proteins has been reported in vivo at the site of inflammatory cell infiltration in disease states associated with epithelial ulceration (11, 18, 45). Studies of mouse small intestinal epithelium in which E-cadherin or β -catenin was overexpressed demonstrated a slowing of normal cell migration from the crypt to the villus (23, 48), indicating that the formation of adherens junctions may correlate with decreased cell migratory ability. In the present study, we observed a decrease in E-cadherin at the periphery of sites of epithelial denudation, consistent with the concept that redistribution

of these proteins away from the leading edge of cells facilitates epithelial migration into the wound.

Our observations suggest that there is limited proteolytic degradation of junctional proteins by neutrophil elastase during PMN transmigration. Exogenous elastase-mediated degradation of the E-cadherin seems to require access of the protease to the basolateral interepithelial space (as evidenced by the requirement of EDTA to open tight junctions). PMN encounter the apical junctional complex during their basolateral to apical migration across epithelium. Local release of PMN proteases may disrupt the junctional complexes by spatially delimited proteolytic degradation mediated in part by leukocyte elastase or other PMN-derived proteases. Alternatively, the effects of PMN elastase could be indirect; elastase could induce changes in epithelial cells leading to activation of alternate proteolytic systems (perhaps epithelium derived) that are the proximate mediators of adherens junction protein degradation. Our experimental system does not allow us to distinguish unequivocally between these possibilities.

Recent investigations of alterations in endothelial β -catenin after PMN adhesion have yielded conflicting results (3, 10, 34). Similar to Moll et al. (34), our laboratory (16) observed that PMN-derived products, likely proteases released after permeabilization, remain active despite exposure to fixatives and may be responsible for an artifactual diffuse degradation of adherens junction proteins. These proteases are resistant to many commonly utilized protease inhibitors (3, 10, 34). By analogy, human or animal tissues fixed by conventional methods that contain infiltrating PMN may well exhibit similar (artifactual) protein degradation. This may complicate the interpretation of reports of loss of adherens junction proteins in conditions in which PMN are present in the tissue, such as inflammatory bowel diseases (11, 18, 45).

In summary, PMN transepithelial migration leads to disruption of the epithelial adherens junctions and detachment of epithelial cells from the monolayer. This is associated with spatially delimited proteolytic degradation of E-cadherin by neutrophil elastase. These data provide a model for the pathogenesis of epithelial ulcerations observed in inflammatory bowel disease, highlight the importance of leukocyte elastase in these processes, and may provide direction for the development of rational therapeutic interventions to ameliorate or prevent this inflammatory epithelial injury.

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REFERENCES

1. Adams CL, Chen YT, Smith SJ, and Nelson WJ. Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *J Cell Biol* 142: 1105–1119, 1998.
2. Adeyemi EO and Hodgson HJ. Faecal elastase reflects disease activity in active ulcerative colitis. *Scand J Gastroenterol* 27: 139–142, 1992.
3. Allport JR, Ding H, Collins T, Gerritsen ME, and Lusinkas FW. Endothelial-dependent mechanisms regulate leukocyte transmigration: a process involving the proteasome and disruption of the vascular endothelial-cadherin complex at endothelial cell-to-cell junctions. *J Exp Med* 186: 517–527, 1997.
4. Bachofen M and Weibel ER. Basic pattern of tissue repair in human lungs following unspecific injury. *Chest, Suppl* 65: 14S–19S, 1974.
5. Bement WM, Forscher P, and Mooseker MS. A novel cytoskeletal structure involved in purse string wound closure and cell polarity maintenance. *J Cell Biol* 121: 565–578, 1993.
6. Cantin AM, Lafrenaye S, and Begin RO. Antineutrophil elastase activity in cystic fibrosis serum. *Pediatr Pulmonol* 11: 249–253, 1991.
7. Castillo MJ, Nakajima K, Zimmerman M, and Powers JC. Sensitive substrates for human leukocyte and porcine pancreatic elastase: a study of the merits of various chromophoric and fluorogenic leaving groups in assays for serine proteases. *Anal Biochem* 99: 53–64, 1979.
8. Champagne B, Tremblay P, Cantin A, and St. Pierre Y. Proteolytic cleavage of ICAM-1 by human neutrophil elastase. *J Immunol* 161: 6398–6405, 1998.
9. Cvetovich RJ, Chartrain M, Hartner FW, Roberge C, Amato JS, and Grabowski EJJ. An asymmetric synthesis of L-694,458, a human leukocyte elastase inhibitor, via novel enzyme resolution of β -lactam esters. *J Org Chem* 61: 6575–6580, 1996.
10. Del Maschio A, Zanetti A, Corada M, Rival Y, Ruco L, Lampugnani MG, and Dejana E. Polymorphonuclear leukocyte adhesion triggers the disorganization of endothelial cell-to-cell adherens junctions. *J Cell Biol* 135: 497–510, 1996.
11. Dogan A, Wang ZD, and Spencer J. E-cadherin expression in intestinal epithelium. *J Clin Pathol* 48: 143–146, 1995.
12. Downey GP, Chan CK, Lea P, Takai A, and Grinstein S. Phorbol ester-induced actin assembly in neutrophils: role of protein kinase C. *J Cell Biol* 116: 695–706, 1992.
13. Downey GP, Chan CK, Trudel S, and Grinstein S. Actin assembly in electroporated neutrophils: role of intracellular calcium. *J Cell Biol* 110: 1975–1982, 1990.
14. Gallin JI, Clark RA, and Kimball HR. Granulocyte chemotaxis: an improved in vitro assay employing ^{51}Cr -labeled granulocytes. *J Immunol* 110: 233–240, 1973.
15. Geiger B and Ayalon O. Cadherins. *Annu Rev Cell Biol* 8: 307–332, 1992.
16. Ginzberg H, Shannon P, and Downey GP. Neutrophil products and alterations in epithelial junctional proteins: prevention of artifactual degradation. *J Immunol Methods* 239: 45–52, 2000.
17. Hampton MB and Winterbourn CC. Modification of neutrophil oxidant production with diphenyleneiodonium and its effect on bacterial killing. *Free Radic Biol Med* 18: 633–639, 1995.
18. Hanby AM, Chinery R, Poulson R, Playford RJ, and Pignatelli M. Downregulation of E-cadherin in the reparative epithelium of the human gastrointestinal tract. *Am J Pathol* 148: 723–729, 1996.
19. Handy LM, Ghosh S, and Ferguson A. Investigation of neutrophils in the gut lumen by assay of granulocyte elastase in whole-gut lavage fluid. *Scand J Gastroenterol* 31: 700–705, 1996.
20. Haslett C, Guthrie LA, Kopaniak MM, Johnston RB Jr, and Henson PM. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol* 119: 101–110, 1985.
21. Heinzelmann M, Mercer-Jones MA, and Passmore JC. Neutrophils and renal failure. *Am J Kidney Dis* 34: 384–399, 1999.
22. Hermiston ML and Gordon JI. Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science* 270: 1203–1207, 1995.
23. Hermiston ML, Wong MH, and Gordon JI. Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system. *Genes Dev* 10: 985–996, 1996.
24. Howard TH and Meyer WH. Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. *J Cell Biol* 98: 1265–1271, 1984.
25. Jaeschke H, Smith CW, Clemens MG, Ganey PE, and Roth RA. Mechanisms of inflammatory liver injury: adhesion molecules and cytotoxicity of neutrophils. *Toxicol Appl Pharmacol* 139: 213–226, 1996.
26. Kondo M, Finkbeiner WE, and Widdicombe JH. Changes in permeability of dog tracheal epithelium in response to hydrostatic pressure. *Am J Physiol Lung Cell Mol Physiol* 262: L176–L182, 1992.
27. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685, 1970.
28. Laskowski M Jr. Protein inhibitors of serine proteinases—mechanism and classification. *Adv Exp Med Biol* 199: 1–17, 1986.
29. Liu L, Mul FPJ, Lutter R, Roos D, and Knol EF. Transmigration of human neutrophils across airway epithelial cell monolayers is preferentially in the physiologic basolateral-to-apical direction. *Am J Respir Cell Mol Biol* 15: 771–780, 1996.
30. Lundqvist H and Dahlgren C. The serine protease inhibitor diisopropylfluorophosphate inhibits neutrophil NADPH-oxidase activity induced by the calcium ionophore ionomycin and serum opsonised yeast particles. *Inflamm Res* 44: 510–517, 1995.
31. MacMicking J, Xie QW, and Nathan C. Nitric oxide and macrophage function. *Annu Rev Immunol* 15: 323–350, 1997.
32. Martin P and Lewis J. Actin cables and epidermal movement in embryonic wound healing. *Nature* 360: 179–183, 1992.
33. Molitoris BA. New insights into the cell biology of ischemic acute renal failure. *J Am Soc Nephrol* 1: 1263–1270, 1991.
34. Moll T, Dejana E, and Vestweber D. In vitro degradation of endothelial catenins by a neutrophil protease. *J Cell Biol* 140: 403–407, 1998.
35. Mumford R, Chabin S, Chiu S, Davies P, Doherty J, Finke P, Fletcher D, Green B, Griffin P, Kissinger A, Knight W, Kostura M, Klatt T, MacCoss M, Meurer R, Miller D, Pacholok S, Poe M, Shah S, Vincent S, Williams H, and Humes J. A cell-penetrant monocyclic β -lactam inhibitor (MBI) which inactivates elastase (E) within PMN in vitro and in vivo (Abstract). *Respir Crit Care Med* 151: A532, 1995.
36. Nagao Y, Miyata H, Mizuguchi K, Ban M, and Kato K. [Effects of ulinastatin on experimental ulcerative colitis in rats]. *Nippon Yakurigaku Zasshi* 109: 41–52, 1997.
37. Nash S, Stafford J, and Madara JL. Effects of polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial monolayers. *J Clin Invest* 80: 1104–1113, 1987.
38. Nash S, Stafford J, and Madara JL. The selective and superoxide-independent disruption of intestinal epithelial tight junctions during leukocyte transmigration. *Lab Invest* 59: 531–537, 1988.
39. Nusrat A, Parkos CA, Liang TW, Carnes DK, and Madara JL. Neutrophil migration across model intestinal epithelia: monolayer disruption and subsequent events in epithelial repair. *Gastroenterology* 113: 1489–1500, 1997.
40. O'Donnell BV, Tew DG, Jones OT, and England PJ. Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem J* 290: 41–49, 1993.
41. Panes J and Granger DN. Leukocyte-endothelial cell interactions: implications for the pathogenesis and treatment of gastrointestinal disease. *Dig Dis* 12: 232–241, 1994.

42. **Parkos CA, Delp C, Arnaout MA, and Madara JL.** Neutrophil migration across a cultured intestinal epithelium. Dependence on a CD11b/CD18-mediated event and enhanced efficiency in physiological direction. *J Clin Invest* 88: 1605–1612, 1991.
43. **Stevenson BR, Siliciano JD, Mooseker MS, and Goodenough DA.** Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol* 103: 755–766, 1986.
44. **Trudel S, Paquet MR, and Grinstein S.** Mechanism of vanadate-induced activation of tyrosine phosphorylation and of the respiratory burst in HL60 cells. Role of reduced oxygen metabolites. *Biochem J* 276: 611–619, 1991.
45. **Valizadeh A, Karayiannakis AJ, el-Hariry I, Kmiot W, and Pignatelli M.** Expression of E-cadherin-associated molecules (α -, β -, and γ -catenins and p120) in colorectal polyps. *Am J Pathol* 150: 1977–1984, 1997.
46. **Vincent SH, Painter SK, Luffer-Atlas D, Karanam BV, McGowan E, Cioffe C, Doss G, and Chiu SH.** Orally active inhibitors of human leukocyte elastase. II. Disposition of L-694,458 in rats and rhesus monkeys. *Drug Metab Dispos* 25: 932–939, 1997.
47. **Waddell TK, Fialkow L, Chan CK, Kishimoto TK, and Downey GP.** Potentiation of the oxidative burst of human neutrophils. A signaling role for L-selectin. *J Biol Chem* 269: 18485–18491, 1994.
48. **Wong MH, Rubinfeld B, and Gordon JI.** Effects of forced expression of an NH₂-terminal truncated β -catenin on mouse intestinal epithelial homeostasis. *J Cell Biol* 141: 765–777, 1998.

