Epithelial Permeability and the Transepithelial Migration of Human Neutrophils

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ABSTRACT Although polymorphonuclear leukocytes (PMN's) can migrate through every epithelium in the body regardless of its permeability, very little is known about the effect of epithelial permeability on PMN migration and the effect of emigrating PMN's on the permeability of the epithelium. In an in vitro model system of transepithelial migration, human PMN's were stimulated by 0.1 μm fMet-Leu-Phe to traverse confluent, polarized canine kidney epithelial monolayers of varying permeabilities. Epithelial permeability was determined by both conductance measurement and horseradish peroxidase (HRP) tracer studies. As epithelial permeability increased, the number of PMN invasion sites as well as the number of PMN's that traversed the monolayer increased. The effect of PMN migration on epithelial permeability was examined using the ultrastructural tracers HRP and lanthanum nitrate. PMN's traversing the monolayer made close cell-to-cell contacts with other invading PMNs and with adjacent epithelial cells. These close contacts appeared to prevent leakage of tracer across invasion sites. Following PMN emigration, epithelial junctional membranes reapproximated and were impermeable to the tracers. These results indicated that, in the absence of serum and connective tissue factors, (a) the number of PMN invasion sites and the number of PMN's that traversed an epithelium were a function of the conductance of the epithelium and (b) PMN's in the process of transepithelial migration maintained close cell-cell contacts and prevented the leakage of particles (>5 nm in diameter) across the invasion site.

Two important characteristics of the acute inflammatory response are an increased microvascular permeability to plasma proteins and the extravascular accumulation of polymorphonuclear leukocytes (PMN's). The permeability and response to inflammation varies in different segments of the microcirculation (11, 29) and with the type and intensity of the stimulus (3). The accumulation of PMN's at the site of inflammation commonly involves the diapedesis of leukocytes across the endothelium of postcapillary venules and frequently the emigration of PMN's across a second epithelium. In fact, PMN's are able to traverse virtually every epithelium in the body (1, 31), regardless of its permeability (8, 14, 25). Yet, little is known about the effect of epithelial permeability on PMN migration or about the effect of PMN migration on the permeability of the epithelium. To study this, we have devised an in vitro system (10) that enables us to examine, in the absence of serum and connective tissue factors, the ability of human PMN's to traverse an epithelium with different permeabilities and the effect of this migration on epithelial permeability. At present, only kidney (6, 24), lung (21), urinary bladder (19), and mam-

mary gland (2) epithelia have been shown to form zonulae occludentes and produce measurable transepithelial electrical resistance when grown in vitro. Since PMN's traverse kidney epithelium in response to infection as well as other pathological conditions (31), we grew a monolayer of canine kidney epithelial cells (MDCK) on micropore filters. The epithelium, whose permeability varied from filter to filter, was used to separate PMN's placed in the upper compartment of a chemotactic chamber from a synthetic chemoattractant in the lower compartment. Under these conditions a chemotactic gradient is established across the filter and PMN's are stimulated to traverse the monolayer. An abstract of this work was previously published (9).

MATERIALS AND METHODS

Preparation of Cells: Madin-Darby canine kidney epithelial cells (MDCK) cells were maintained in culture by serial passage in Eagle's medium with Earle's salts, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% (vol/vol) fetal calf serum. The cells were harvested with 0.25% trypsin/2 mM EDTA in Dulbecco's phosphate-buffered saline (Ca²⁺- and Mg²⁺-free) and plated (1–4 × 10⁵ cells/ml) on 13-mm diameter, 0.45-μm pore-size micropore filters (Milli-

pore/Continental Water Systems, Bedford, MA). All tissue culture materials were obtained from Gibco Laboratories (Grand Island, NY).

Human PMN's were isolated from citrated venous blood from normal human volunteers. The blood was separated into a granulocyte-rich fraction by Hypaque/ Ficoll and dextran sedimentation techniques (4). Residual erythrocytes were eliminated by hypotonic lysis with 0.2% sodium chloride. This resulted in a cell fraction containing 98% PMN's with 98–99% viability as determined by trypan blue dye exclusion. Before use, PMN's were suspended at 5×10^6 cells/ml in Gey's balanced salt solution (Geys; Microbiological Associates, Walkersville, MD; 294 mosM/kg) containing 2% (wt/vol) bovine serum albumin, penicillin (63 U/ml), and streptomycin (138 μg/ml).

Conductance Measurements: As a test of the confluence of the MDCK monolayer, transepithelial electrical resistance studies (23) were performed before all experiments. The MDCK monolayers grown on micropore filters were placed between the two halves of a Ussing chamber exposing 0.32 cm^2 of the monolayer. 10 μA of current was passed across the monolayer through Hg/HgCl electrodes connected to the chamber with 3 M KCl-agar bridges. The voltage deflection measured on a Keithley 610C electrometer (Keithley Instruments, Inc., Cleveland, OH) was used to calculate the transepithelial electrical resistance. Conductance, a direct measure of the permeability of the epithelial junctions, was calculated as the inverse of the resistance. All measurements were done at room temperature in modified Eagle's medium containing 10% (vol/vol) fetal calf serum.

Transepithelial Neutrophil Migration: After conductance was measured, MDCK monolayers grown on 0.45-μm Millipore filters (Millipore/ Continental Water Systems) were washed in Geys and then placed in a Boyden chemotactic chamber (Neuroprobe, Bethesda, MD) with the epithelium facing the upper compartment. PMN's were stimulated to traverse the epithelial monolayer from the apical surface over a 45-min incubation period by 0.1 μM fMet-Leu-Phe (courtesy of Elliot Schiffmann, National Institute of Dental Research, or from Peninsula Laboratories, San Carlos, CA) in the lower compartment. All transepithelial migration studies were done at 37°C in a 95% air/5% CO$_2$ atmosphere. The epithelial monolayers were then fixed and embedded in Epon 812 for electron microscopy (see below).

Quantitative Measurements of PMN Migration and Invasion Sites: Two random sections (5–7 mm long) of the epithelial monolayer (8-mm diameter) were taken from each experimental filter. 1-μm Epon sections were stained with toluidine blue and examined by light microscopy. PMN's that had traversed the epithelial monolayer were caught beneath the epithelium at the surface of the small pore-size filter. They were counted in both sections and the number was expressed as the number of PMN's/mm epithelium per filter. The number of PMN invasion sites was determined by counting the number of discrete clusters of PMN's that accumulated beneath an epithelial junction. These numbers were expressed as the number of invasion sites/mm epithelium.

Ultrastructural Tracer Studies: The ultrastructural tracer, horseradish peroxidase (HRP, type II, EC1.11.1.7; Sigma Chemical Co., St. Louis, MO) was used to assess the permeability of individual epithelial junctions as well as the permeability of PMN invasion sites. HRP produced an electron-dense reaction product that filled the intercellular space and indicated permeable and impermeable junctions or regions only when it diffused across the epithelium from the basal direction. It was not a useful ultrastructural tracer when added to the apical surface of the epithelium because it did not form a continuous electron-dense deposition on the surface of the monolayer and in this situation did not clearly demonstrate permeable and impermeable areas. As a result, HRP (185.6 U/mg per ml) alone or added to 0.1 μM fMet-Leu-Phe in Geys (295 mosM/kg) was placed beneath the epithelium in the lower compartment of the Boyden chamber. In some experiments, PMN's were added to the upper compartment. After a 45-min incubation the solutions were removed and the epithelial monolayer with or without PMN's was fixed in the chemotactic chamber for 1 h with 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0, 495 mosM/kg) placed in both compartments. The monolayer was then washed in 0.1 M phosphate buffer (pH 6.5), removed from the chamber, and processed for peroxidase localization by incubating in 1.96 mg of 3,3'-diaminobenzidine tetrahydrochloride (Polysciences, Inc., Warrington, PA) per ml of 0.1 M phosphate buffer (pH 6.5) containing 0.02% (vol/vol) H$_2$O$_2$ (3%; Fisher Scientific Co., Allied Corp., Pittsburgh, PA; reference 15). After a 20-min incubation the monolayer was postfixed in 1% (vol/vol) OsO$_4$ in 0.1 M phosphate buffer (pH 7.3) for 1 h and then dehydrated and embedded in Epon 812. Ultrathin sections were cut on an LKB Ultrotome III (LKB Instruments, Inc., Gaithersburg, MD) and examined with the JEOL 100C electron microscope. The percentage of junctions permeable to HRP was determined from three to five ultrathin sections taken at intervals of >100 μm. At least 100 junctions from each monolayer were counted.

Lanthanum nitrate tracer studies were performed to assess the permeability of PMN invasion sites from the apical direction. The osmolarity as determined by freezing point depression in a Precision osmometer (VWR Scientific Inc., San Francisco, CA) and the concentration of the lanthanum nitrate solutions were found to be very important. When the osmolarity increased above 700 mosM/kg,

the tracer leaked across every junction. All lanthanum studies were done after PMN's were stimulated to traverse an epithelial monolayer for 45 min. Lanthanum nitrate (13.7 mM; Fisher Scientific Co.) in 2% (vol/vol) glutaraldehyde-0.1 M sodium cacodylate buffer (pH 7.8, 458 mosM/kg) was placed in the upper compartment while the same fixative without lanthanum (433 mosM/kg) was placed in the lower compartment for 2 h at room temperature. The epithelial monolayers with invading PMN's were then washed overnight in 0.2 M phosphate buffer (pH 7.4) at 4°C to precipitate the lanthanum (27). It is not known whether the precipitate is due to the ionic or colloidal form of lanthanum (26). Postfixation, dehydration, and embedding were carried out as described above.

RESULTS

Epithelial Permeability

After 2–3 d, MDCK cells plated on micropore filters formed confluent, polarized monolayers with zonulae occludentes (tight or occluding junctions) at their apical border. Confluent monolayers exhibited a natural variation in their conductance ranging from 2.5 to 9.5 mS/cm^2. While conductance studies gave a measure of the permeability of the entire monolayer, HRP tracer studies provided a means to assess the permeability of individual junctions. Using the electron microscope, we examined the diffusion of tracer across 16 monolayers with conductances (or permeabilities) increasing from 2.5 to 8.2 mS/cm^2 (Fig. 1). At conductances of 6.7 mS/cm^2, at least 99% of the epithelial junctions excluded HRP. However, when the permeability of the monolayer increased above 6.7 mS/cm^2, there was a progressive increase in the number of junctions permeable to the tracer. This reached a maximum at 8.2 mS/cm^2 when ~7% of the junctions were permeable to HRP.

Effect of Epithelial Permeability on Neutrophil Migration

We took advantage of the natural variation in the permeability of the epithelium and examined the number of PMN invasion sites as well as the number of PMN's that had emigrated through 27 monolayers (5.1–9.5 mS/cm^2) under conditions of chemotaxis (buffer in upper compartment, 0.1 μM fMet-Leu-Phe in lower compartment).

There was a striking difference in the number of PMN invasion sites at various conductances. This can be seen in Fig. 2. In highly permeable monolayers, PMN's traversed the epi-
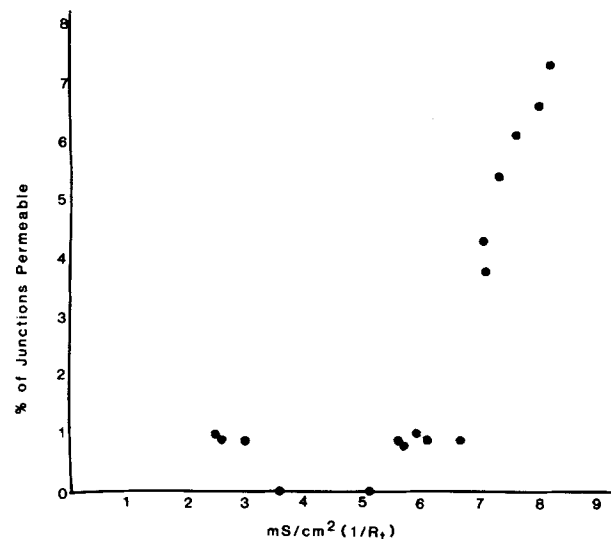

FIGURE 1 The percent of junctions permeated by HRP was correlated with the conductance (mS/cm^2, 1/ R_t) of the monolayer. Each point represents at least 100 junctions from one monolayer.
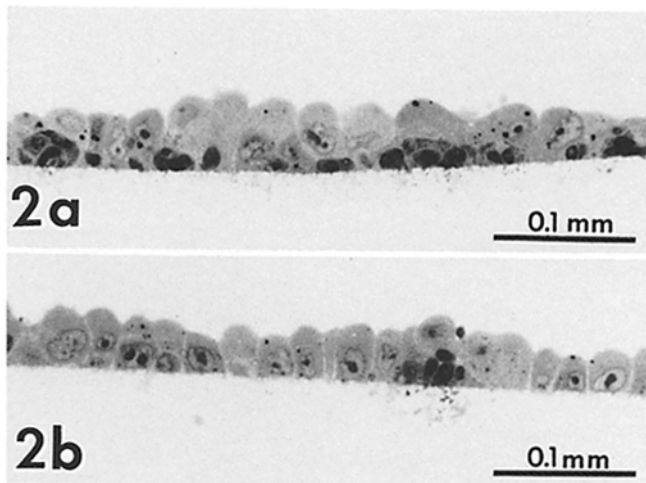
FIGURE 2 Transepithelial migration of human PMN's across (a) low (5.4 mS/cm^2) and (b) high (9.9 mS/cm^2) conductance monolayers. Note the decrease in both the number of emigrated PMN's (dark nuclei) and the number of PMN invasion sites in the high conductance monolayer (b). Bars, 0.1 mm. Stained with toluidine blue; × 530.

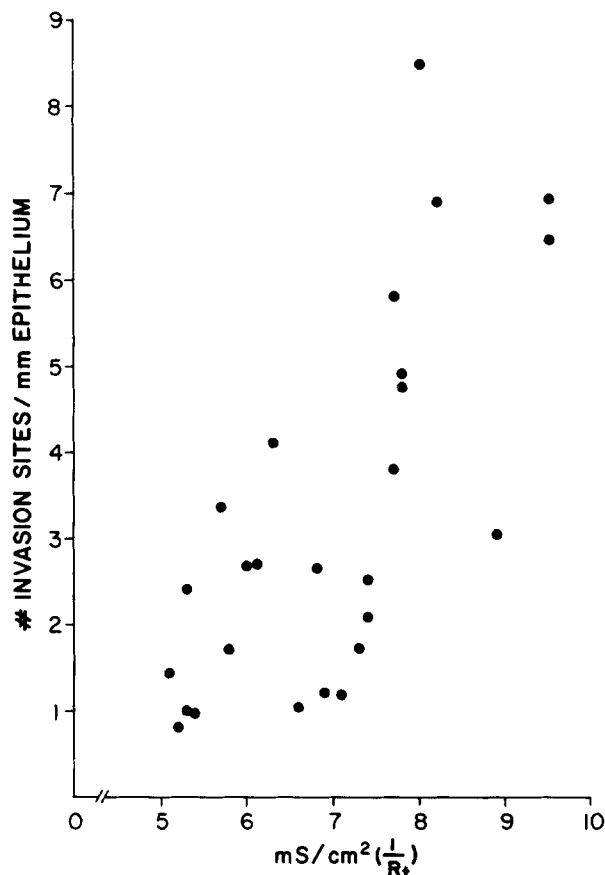FIGURE 3 The number of PMN invasion sites/mm epithelium was positively correlated (r = 0.72, P < 0.001) with the conductance (mS/cm^2, 1/ R_t) of the monolayer. Each point represents the average number of PMN invasion sites/mm epithelium of two, 5-7-mm-length segments of the epithelial monolayer.

FIGURE 4 The number of emigrated PMN's/mm of epithelium was positively correlated (r = 0.58, P < 0.01) with the conductance (mS/cm^2, 1/ R_t) of the monolayer. Each point represents the average number of PMN's/mm of epithelium that traversed two, 5-7-mm-length segments of the epithelial monolayer.

conductance of the monolayer (Fig. 3), a significant correlation coefficient (r = 0.7, P < 0.001) was obtained. Similarly, a significant correlation (r = 0.5, P < 0.01) was also observed when the number of PMN's that traversed the epithelium was compared to the conductance of the monolayer (Fig. 4). As one might expect, the number of PMN's that migrated across the epithelium was a function (r = 0.8, P < 0.001) of the number of PMN invasion sites. On the average, as determined by the method of linear regression, ~2 PMN's migrated across each invasion site in 45 min. Therefore, when the permeability of the epithelium decreased, the number of PMN invasion sites decreased and, consequently, the number of PMN's which migrated across the epithelial monolayer also decreased.

Effect of Neutrophil Migration on Epithelial Permeability

Ultrastructural tracers were used to assess the permeability of epithelial junctions during the process of transepithelial migration of PMN's. When PMN's were observed adherent to the surface above epithelial junctions, the junctions were not permeated by HRP (Fig. 5) or lanthanum nitrate. PMN's in the process of traversing the monolayer formed regions of close cell-cell contacts between themselves and the epithelium. These contacts prevented the passage of tracers from either the basal (HRP; Fig. 6) or apical (lanthanum nitrate; Fig. 7) direction.

thelium at many junctional sites (Fig. 2 a). In contrast, in monolayers with lower conductances, PMN's penetrated fewer epithelial junctions and clustered beneath the epithelium in isolated areas (Fig. 2 b). When the number of PMN invasion sites/mm of epithelium was counted and correlated with the
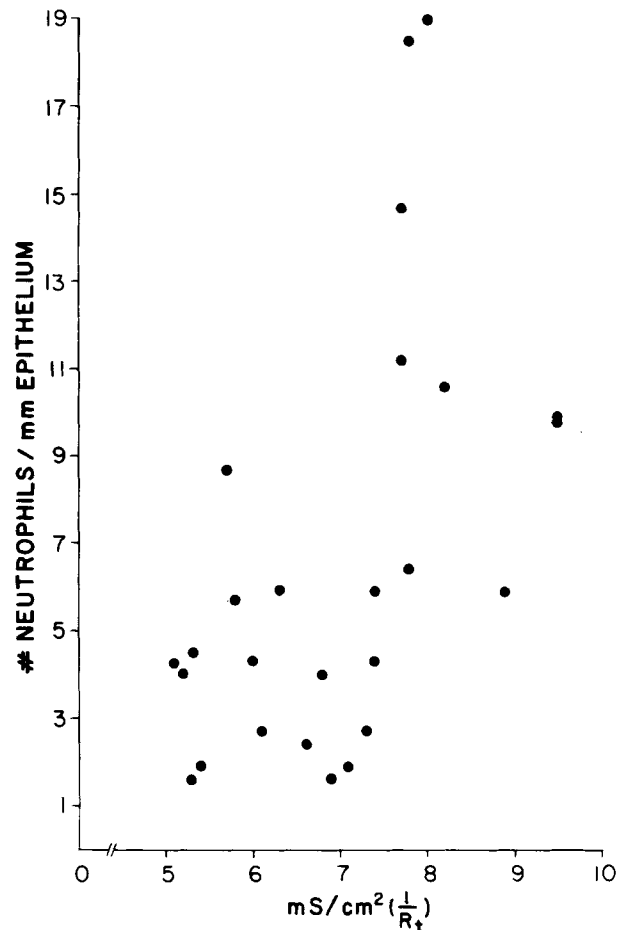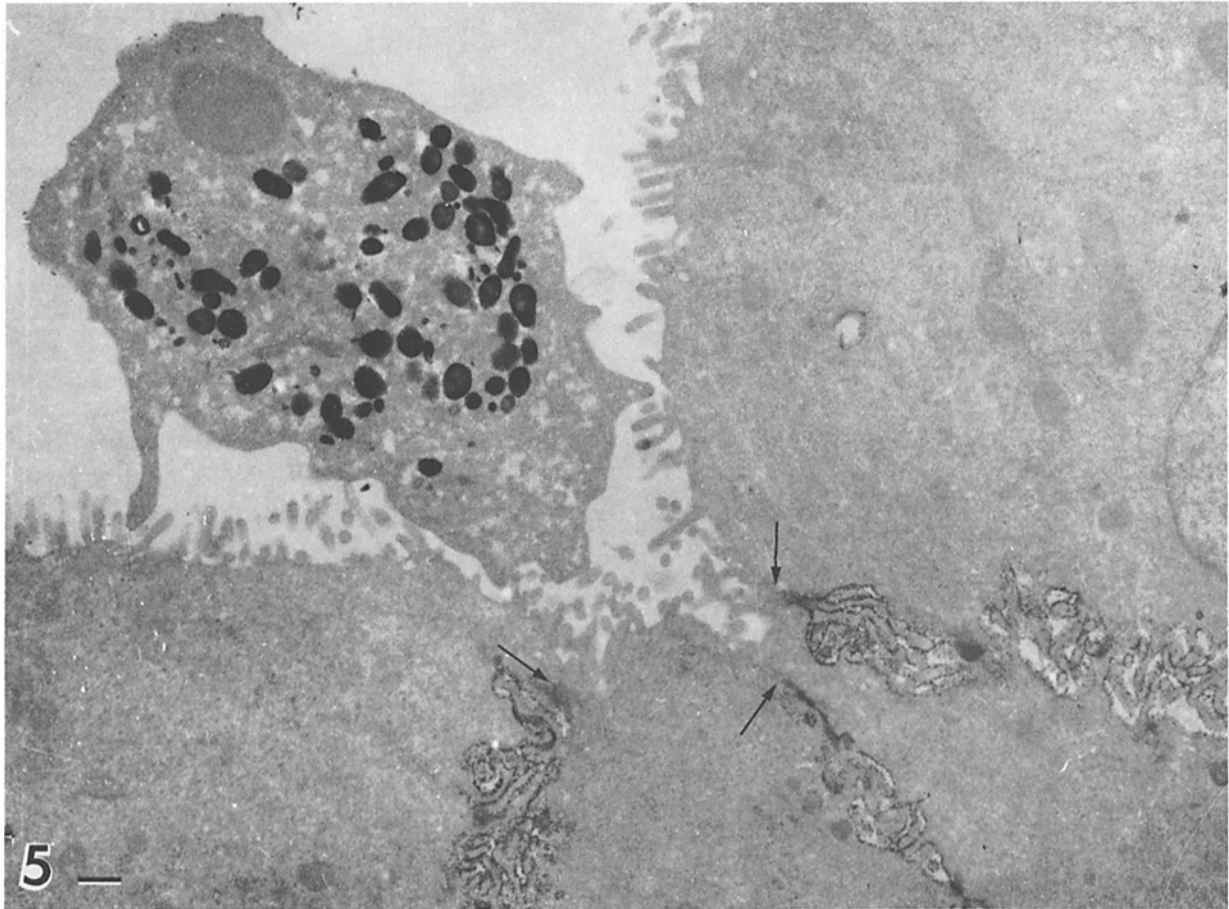
FIGURE 5   Transmission electron micrograph of a PMN oriented above four adjacent epithelial cells. HRP was placed beneath the epithelial monolayer with the chemoattractant fMet-Leu-Phe for 45 min. HRP reaction product is observed in the lateral intercellular spaces between epithelial cells but is excluded from the occluding junctions (arrows). Bar, 1.0 μm. Unstained; × 18,900.

Following PMN emigration, the epithelial junctional membranes reapproximated and were impermeable to both HRP (Fig. 8) and lanthanum nitrate. The entire process of PMN transepithelial migration and resealing of the epithelial junction occurred within 45 min.

DISCUSSION

The results of our study indicate that the number of PMN's that traverse an epithelial monolayer is dependent on the number of PMN invasion sites and the conductance or ionic permeability of the epithelium. As the conductance decreases, the number of junctions penetrated by PMN's and thus, the number of emigrated PMN's, decreases. These findings suggest and support the observation of Cereijido and his collaborators (7) that the permeability of occluding junctions within a monolayer is not uniform and that when the conductance of the monolayer decreases the number of high permeability sites decreases. A decrease in permeability of individual junctions with decreasing epithelial conductance is also indicated by our HRP study. As the conductance of the monolayers decreases from 8.2 to 6.7 mS/cm^2, the number of junctions permeated by HRP (40,000 mol wt, 5-nm diameter, reference 28) decreases. However, at conductances below 6.7 mS/cm^2, 99–100% of the junctions exclude HRP, and the tracer is no longer effective in distinguishing the more subtle differences in junctional permeability. On the other hand, the number of PMN invasion sites continues to decline in monolayers with conductances below

6.7 mS/cm^2. The average number of invasion sites in monolayers with conductances between 5.0 and 5.9 mS/cm^2 is 35% lower than in monolayers with conductances between 6.0 and 6.7 mS/cm^2. Thus it appears that one of the limiting factors in transepithelial migration of human PMN's is the tightness of the occluding junction. It is not possible to distinguish whether this limitation is due to the inability of chemoattractant to diffuse across the epithelium and/or the inability of the PMN's to penetrate the tighter junctions. However, since PMN's in vivo are capable of migrating across epithelia with conductances 10 to 20 times lower than those recorded in our in vitro system (1, 8, 14, 25, 31), it would appear that other factors, perhaps serum or connective tissue components, modulate this process. The identification of these factors and their role remains to be elucidated.

The results of the second part of this study support the notion that the integrity of the epithelial monolayer is maintained during the emigration of PMN's. The method by which PMN's penetrate the zonula occludens is unknown. Whether the process involves any of the known means of opening tight junctions such as proteases, hypertonicity, change in pH, disruption of epithelial microfilaments, removal of extracellular calcium ions, or an increase in epithelial calcium concentration remains to be determined (5, 22). We did not detect loosening of the zonulae occludentes (indicated by leakage of tracer across the epithelium) when PMN's were adherent to the epithelial surface before their penetration of the junction. As
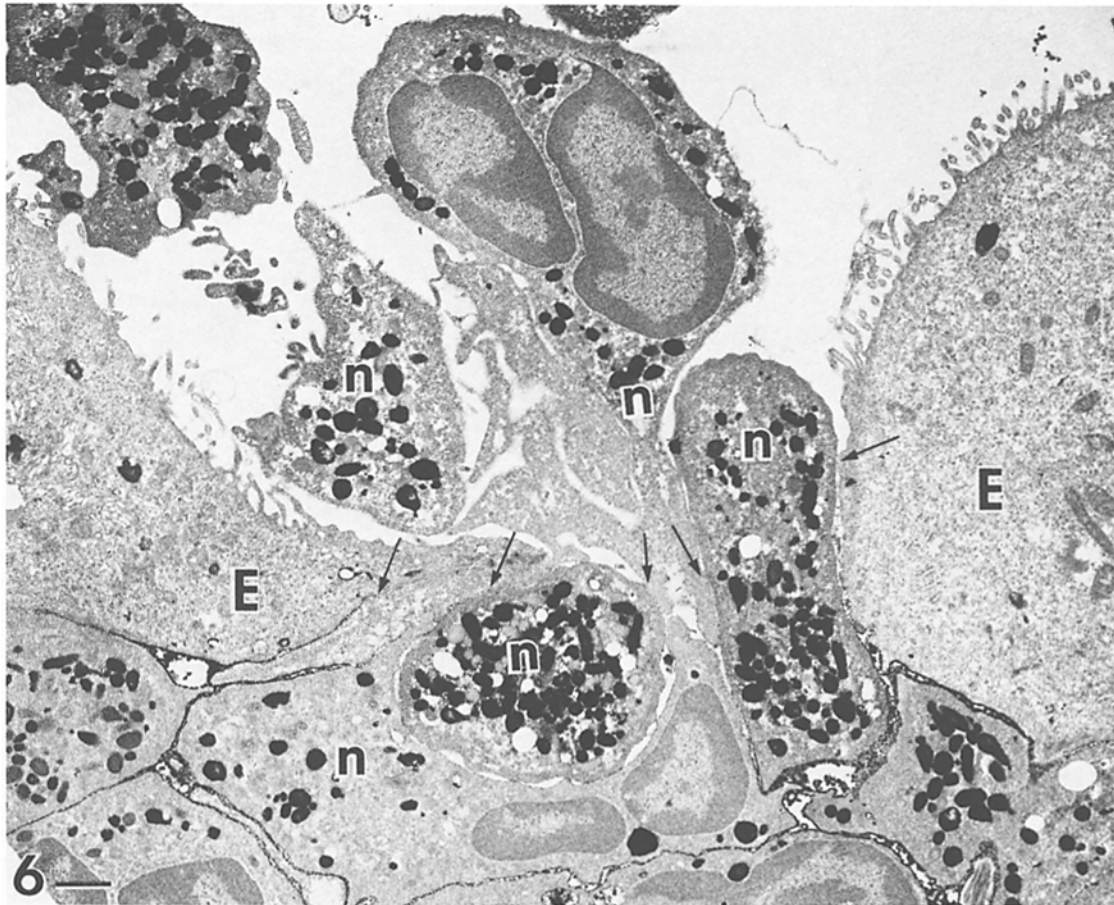
FIGURE 6 Transmission electron micrograph of a PMN invasion site. HRP was placed beneath the epithelial monolayer (*E*) with the chemoattractant fMet-Leu-Phe for 45 min. The electron-dense reaction product surrounds the emigrated PMN's (*n*) and is prevented from passing across the invasion site at the arrows. Bar, 1.0 μm. Unstained; × 13,900.

PMN's migrate through the epithelial junction they make close cell-cell contacts with other PMN's and with adjacent epithelial cells. These contacts appear to prevent leakage of either HRP or lanthanum nitrate across the invasion site. Following PMN emigration, the junctional membranes rapidly reapproximate (within 45 min) and are impermeable to tracer (Fig. 8). Therefore, the method that PMN's use to penetrate occluding junctions probably does not disrupt the junctions to the extent that extensive *de novo* protein synthesis is necessary for junctional repair. This would be consistent with the observation that protein synthesis is not required for the reassembly of junctions in MDCK monolayers treated with EGTA (13, 20). While tracer studies provide some indication of the permeability of junctions during transepithelial migration of PMN's, they do not indicate whether the ion permeability of the epithelium is maintained. Conductance measurements during PMN emigration are in progress.

And, finally, the results of our in vitro study lend support to the concept that, during an acute inflammatory reaction, leukocytic emigration and an increase in vascular permeability may sometimes be separate phenomena (16, 17). However, PMN emigration and an increase in vascular permeability can co-exist in a single vessel (17). The factors that control this reaction are the source of recent in vivo studies (12, 18, 30). It is clear that the chemoattractants fMet-Leu-Phe (30) and leukotriene B₄ (12) alone cause little increase in vascular permeability. The situations in which PMN migrations are associated with increases in permeability appear to be due to synergistic effects of leukocytes, serum, and connective tissue components and endothelium. Through the use of an in vitro model system, it may be possible to more easily separate the roles of these factors and to provide another means of examining the inflammatory response.
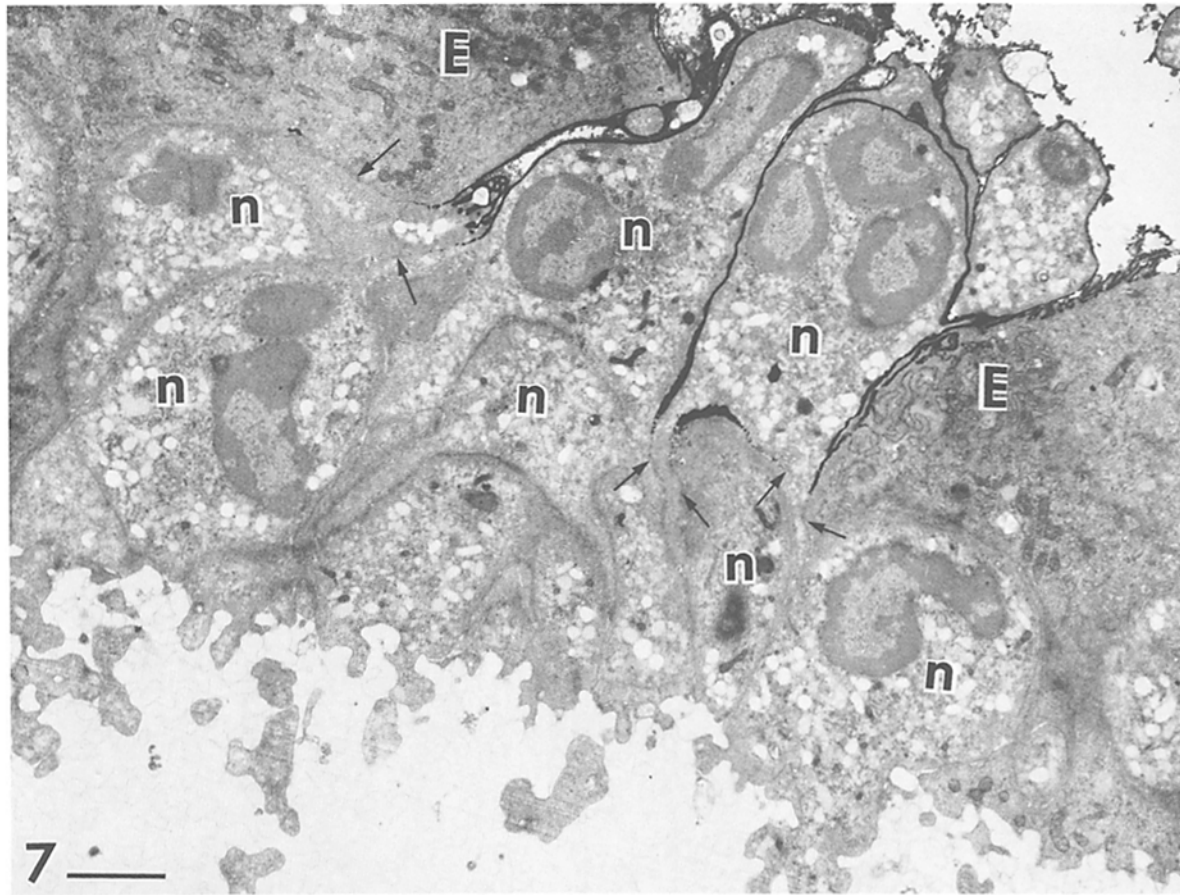
FIGURE 7 Transmission electron micrograph of a PMN invasion site. Lanthanum nitrate was placed with the fixative above the epithelial monolayer (E). The electron-opaque tracer covers the apical surface of the invading PMN's (n). The tracer is prevented from traversing the invasion site at the arrows. 7,600. Bar, 1.0 µm. Unstained; × 7,600.
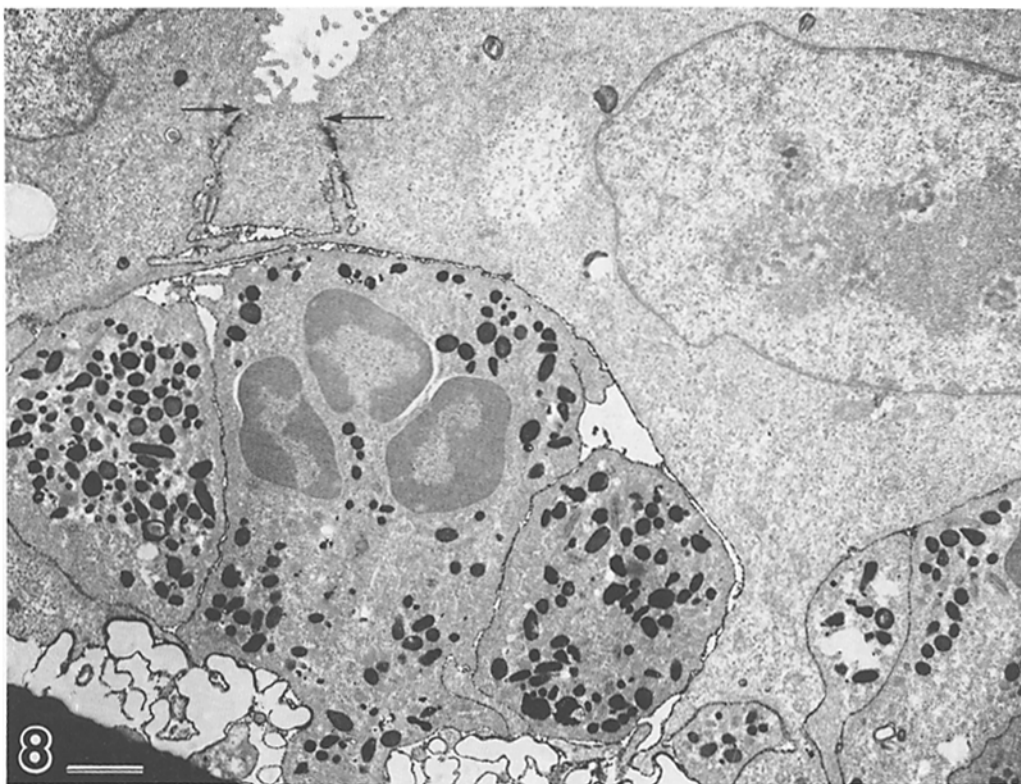

FIGURE 8 Transmission electron micrograph of PMN's that have emigrated across an epithelial monolayer. HRP was placed with the chemoattractant beneath the epithelium for 45 min. The electron-dense reaction product surrounds the emigrated PMN's, fills the lateral intercellular spaces between the epithelial cells, but is excluded from the reapproximated junctional membranes (arrows). Scale Bar, 1.0 µm. Unstained; × 9,800.

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