

The Effect of Neutrophil Migration on Epithelial Permeability

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Abstract. To reach an inflammatory lesion, neutrophils must frequently traverse the epithelium of an infected organ. Whether the actual migration of neutrophils alters the epithelial permeability is unknown. Through the use of an *in vitro* model system it was possible to directly determine the effect of neutrophil emigration on the transepithelial electrical resistance of the monolayer. Human neutrophils (5×10^6 cells/ml) were placed in the upper compartment of a combined chemotaxis/resistance chamber and stimulated for 40 min by a gradient of 10^{-7} M *n*-formyl-methionyl-leucyl-phenylalanine to traverse a confluent monolayer of canine kidney epithelial cells grown on micropore filters. Neither the chemoattractant alone (10^{-5} – 10^{-9} M) nor the accumulation of an average of eight neutrophils per millimeter of epithelium lowered the transepithelial electrical resistance. However, under certain conditions the migration of neutrophils temporarily increased the permeability of the monolayer.

The resistance fell $\sim 48\%$ within 5 min if the migratory cells were stimulated to reverse their migration across the same monolayer. As re-migration continued, the resistance returned to its initial levels within 60 min. Doubling the initial neutrophil concentration to 10×10^6 cells/ml resulted in the accumulation of an average of 66 neutrophils per millimeter of epithelium and an average fall in resistance of 46% ($r = 0.98$; $P < 0.001$) in 40 min. If the resistance had fallen $<45\%$, removal of the neutrophils remaining in the upper compartment resulted in a return of the transepithelial electrical resistance to its initial level within 65 min. However, when the fall was $>45\%$, the resistance only recovered to 23.5% of its initial levels within the same time frame. Thus, these results suggest that the integrity of an epithelium can, under certain conditions, be affected by the emigration of neutrophils, but that this effect is either completely or partially reversible within 65 min.

THE accumulation of leukocytes is an important part of the inflammatory response. To reach the site of inflammation, leukocytes must be able to traverse the endothelium lining blood vessels and the epithelium lining an infected organ (i.e., transitional epithelium during a bladder infection or tubular epithelium in pyelonephritis; 9, 24, 40, 41). While many *in vivo* studies have examined the effect of leukocyte migration on the permeability of the vascular endothelium (1, 18–22, 26–28, 34, 39, 42, 44, 46), relatively few studies (8, 36, 43, 45) have determined the effect of inflammatory mediators or neutrophil migration on the permeability of organ epithelia.

The paracellular or intercellular permeability of the epithelium is regulated by the zonulae occludentes (tight or occluding junctions; 5, 11). Ultrastructurally, these junctions appear as regions of fusion between the outer leaflets of the plasma membrane of adjacent epithelial cells and are thought to form an occluding belt around the circumference of each cell (3, 10). The tightness of these junctions can be assessed by transepithelial electrical resistance measurements (11, 37).

When leukocytes traverse epithelia, they must migrate either through the cells or between the occluding junctions. From previous studies in our laboratory (6, 7, 36) as well as

work by others (8, 24), neutrophils have been shown to migrate between epithelial cells. The effect of this process on the transepithelial electrical resistance of the epithelium is unknown. To determine the effect of leukocyte migration on epithelial permeability we have used an *in vitro* model of neutrophil transepithelial migration (6). With this system a confluent polarized monolayer of Madin-Darby canine kidney (MDCK)¹ epithelial cells are grown on micropore filters. Transepithelial electrical resistance measurements were monitored continuously as human neutrophils traversed the epithelium in response to the chemoattractant, *n*-formyl-methionyl-leucyl-phenylalanine (fMLP). The effect of the chemoattractant, neutrophil concentration, and the repeated migration of the same cells across the same epithelium on the permeability of an epithelial monolayer was assessed.

A preliminary report of portions of this work has already been published (35).

1. *Abbreviations used in this paper:* CMEM, MEM with 10% vol/vol fetal calf serum; fMLP, *n*-formyl-methionyl-leucyl-phenylalanine; Gey's, Gey's balanced salt solution containing 0.5% wt/vol bovine serum albumin; MDCK, Madin-Darby canine kidney epithelial cells.

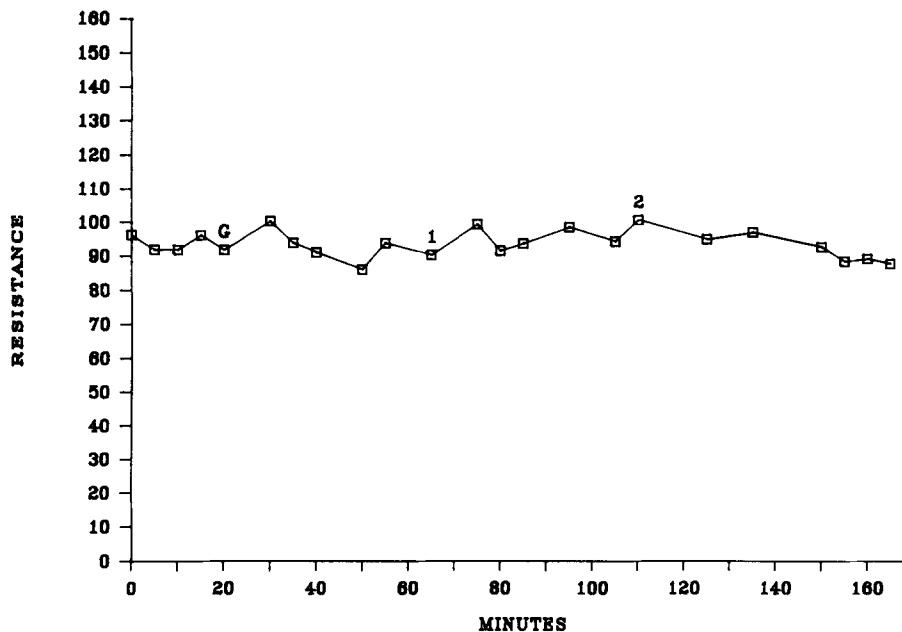


Figure 1. Continuous transepithelial electrical resistance recording of a control MDCK monolayer illustrating the effect of changing the Gey's buffer (G) or adding 10^{-7} M fMLP either to the lower (chemotaxis; 1) or to both compartments (chemokinesis; 2) for 40 min at 37°C .

Materials and Methods

Epithelial Cell Culture

MDCK epithelial cells were maintained in culture by serial passage in Eagle's medium with Earle's salts (MEM), penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and 10% vol/vol fetal calf serum (CMEM), as described previously (6). For experimental procedures, micropore filters of varying pore size (3.0 or 0.45 μm), and composition (mixed cellulose acetate and nitrate, Millipore Corp., Bedford, MA, or cellulose nitrate, Sartorius Filters, Inc., Hayward, CA) were glued (silicone adhesive/sealant, Dow Corning Corp., Midland, MI) to the lower end of rimmed hollow plastic cylinders (15, 33). The epithelial cells were plated inside the cylinder on the filter surface (area: 0.71 cm^2) at a density between $1-2 \times 10^5$ cells/ cm^2 . The cylinders were suspended in a 24-multiwell tissue culture plate for 2-4 d with media changed daily. At confluency there were $\sim 4 \times 10^5$ cells on the filter surface. All tissue culture materials were obtained from Gibco (Grand Island, NY) and culture plasticware from Falcon Labware (Oxnard, CA).

Neutrophil Isolation

Human neutrophils were isolated from citrated venous blood using the Hypaque/Ficoll and dextran sedimentation techniques (2) followed by hypotonic lysis. This resulted in a cell fraction containing 98% neutrophils with 98-99% viability as determined by trypan blue dye exclusion (38). Before use, neutrophils were suspended at either 5 or 10×10^6 cells/ml in either MEM, CMEM, or Gey's balanced salt solution (Gey's; Gibco) containing 0.5% wt/vol bovine serum albumin (Sigma Chemical Co., St. Louis, MO).

Transepithelial Electrical Resistance Measurements

As a test of the confluence of the epithelial monolayer, the transepithelial electrical resistance (37) was measured for each filter before all experiments. Epithelial cells grown on micropore filters glued to cylinders were measured in a specially designed vertical chamber (Scientific and Medical Instrumentation Center, State University of New York at Downstate Medical Center, Brooklyn, NY) which permitted simultaneous electrical resistance measurements and leukocyte chemotaxis studies. 10 μA of direct current were passed across the cell monolayer through Hg/HgCl₂ electrodes connected to each compartment by 3 M KCl-agar bridges (0.042" i.d.). The voltage change was measured on a digital multimeter (model 191; Keithley Instruments, Inc., Cleveland, OH), and this measurement was used to calculate the transepithelial electrical resistance of the epithelial monolayer. The presence of the 3 M KCl bridges caused the level of extracellular potassium in the MEM to rise from 5.3 ± 0.3 to 9.97 ± 0.5 meq/liter in the apical compartment and 7.2 ± 0.3 meq/liter in the basal compartment after 40 min. This increase did not affect the stability of the transepithelial electrical

resistance of the monolayers. All readings were done at 37°C in a 95% air/5% CO₂ incubator. Each monolayer was allowed to stabilize in the chamber and in the buffer used for the experiment (CMEM, MEM, or Gey's) for at least 15 min. Monolayers with starting resistances <95 ohms- cm^2 were found to vary in their stability, therefore only monolayers with resistances at or greater than this level were used for these studies. In analyzing some of the data, the resistance measurements of the monolayers were normalized to their starting resistance. The mean and SEM were then calculated. Statistical differences between control and experimental groups were tested at the 40 (± 2) min timepoint using Student's *t* test.

Migration Studies

Neutrophils (0.5 ml), at concentrations of either 5 or 10×10^6 cells/ml, were placed above monolayers grown on 0.45- or 3.0- μm pore filters. The cells were stimulated to traverse the epithelium by 10^{-7} fMLP (Peninsula Laboratories, San Carlos, CA), a synthetic chemotactic peptide, placed in the compartment below the filter. The temperature and pH were maintained by placing the chamber in an air/CO₂ incubator (Forma Scientific, Marietta, OH). The transepithelial electrical resistance of the monolayer was monitored throughout the incubation period. After 5, 10, 15, 30 or 40 min, the filters were processed for light, transmission, or scanning electron microscopy (see below). The number of neutrophils that traversed the monolayer per millimeter of epithelium was quantitated from 1- μm sections as previously described (6). Neutrophil migration began after 5 min of incubation.

The viability of the epithelial monolayers after neutrophil migration was determined by the ability of the epithelial cells to exclude trypan blue (38). The filters were placed in 0.08% trypan blue (0.4% diluted 1:4; Gibco) for ~ 1 min, fixed in absolute methanol for 15 s, then rapidly dehydrated in ethanol, cleared in xylene, and mounted in Permount. The number of damaged or dead cells was visually determined by light microscopy.

Preparation for Electron Microscopy

Micropore filters containing both neutrophils and epithelial cells from both experimental and control groups were fixed for 1 h in 2.5% vol/vol glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), washed in buffer, and then processed for transmission and/or scanning electron microscopy. For transmission electron microscopy, the filters were postfixated for 1 h in 1% vol/vol OsO₄ in 0.1 M phosphate buffer (pH 7.3), washed in 0.85% saline, dehydrated in ethanol, and embedded in Epon 812. Thin sections, stained with uranyl acetate and lead citrate, were examined with the JEOL 100C electron microscope. For scanning electron microscopy, the filters were impregnated with OsO₄ using the thiocarbonylhydrazide/osmium tetroxide technique (30), dehydrated in ethanol, critical-point dried (Samdri 790; Tousimis Research Corp., Rockville, MD), and examined with the JEOL 100C in the scanning mode.

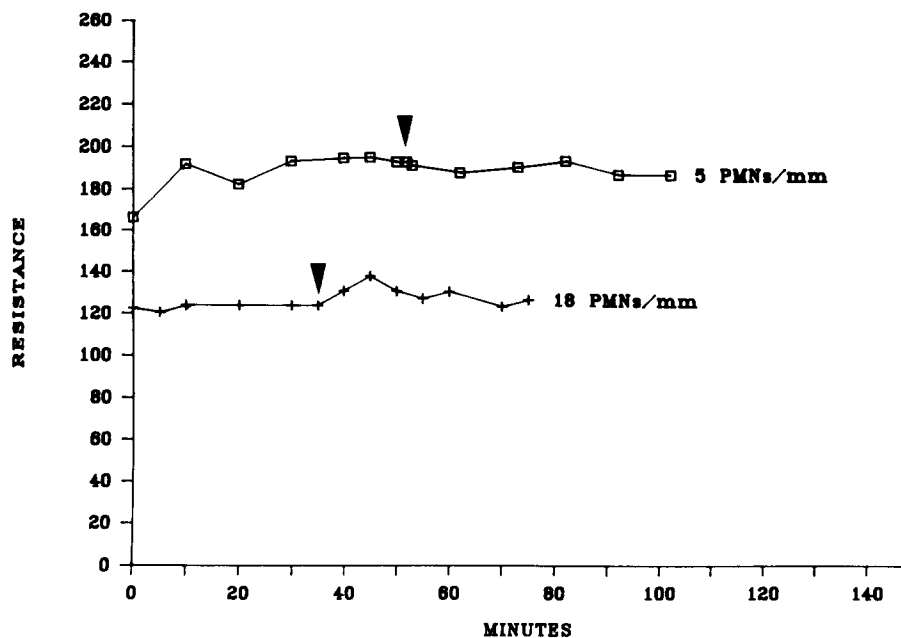


Figure 2. Effect of neutrophil migration on the transepithelial electrical resistance. Neutrophils at a concentration of 5×10^6 neutrophils/ml were added to the apical compartment and stimulated to traverse the monolayer by 10^{-7} M fMLP placed in the basal compartment at the time indicated by the arrowhead. Continuous resistance measurements were taken during the 40-min incubation period. The resistance remained stable regardless of the starting resistance of the monolayer or whether up to 18 neutrophils traversed per millimeter of epithelium.

Results

Effect of Media and Chemoattractant on the Permeability of Epithelial Monolayers

Under control conditions (media on both sides of the monolayer, no neutrophils or chemoattractant present) the transepithelial electrical resistance of epithelial monolayers remained relatively stable for 40–45 min. As seen in Fig. 1, neither the changing of solutions nor the addition of the chemoattractant (10^{-7} M fMLP) to either the lower or both compartments altered the epithelial resistance. In fact, fMLP at all concentrations (10^{-5} – 10^{-9} M) tested had no significant effect on the transepithelial electrical resistance of the monolayer (data not shown). Most studies were performed in Gey's with 0.5% BSA. However, similar results were observed when experiments were performed in media with (CMEM) or without (MEM) 10% fetal bovine serum. All cells were viable as determined by trypan blue exclusion even after continuous transepithelial electrical resistance measurements lasting 180 min or after a 40-min exposure to a high concentration (10^{-5} M) of the chemoattractant.

Continuous Resistance Measurements during Neutrophil Migration

Continuous transepithelial electrical resistance studies were performed during neutrophil migration. Neutrophils suspended at a concentration of 5×10^6 cells/ml were placed above the apical epithelial surface. They were stimulated to traverse the epithelia grown on 0.45- μ m pore micropore filters by 10^{-7} M fMLP, placed in the lower compartment. As can be seen in Fig. 2, the resistance remained stable throughout the \sim 40-min incubation period. At the end of the incubation the filters were fixed, embedded, and the number of neutrophils that traversed each monolayer per millimeter of epithelium was determined. As previously observed (36), the number of neutrophils that traversed the monolayer was dependent on the initial transepithelial electrical resistance

of the monolayer. Consequently, more neutrophils traversed epithelia with lower starting resistances. However, even when as many as 18 neutrophils per millimeter of epithelium had traversed the monolayer, the resistance remained stable (Fig. 2).

Effect of Neutrophil Concentration on Epithelial Permeability

To determine whether the concentration of neutrophils stimulated to traverse the epithelium had any effect on the permeability of the monolayer, neutrophils were isolated from the same donor, suspended at concentrations of 5 and 10×10^6 neutrophils/ml, and stimulated to migrate across epithelial monolayers with comparable transepithelial electrical resistances (Fig. 3). While the resistance of the monolayer remained stable when a concentration of 5×10^6 neutrophils/ml was used, a concentration of 10×10^6 neutrophils/ml caused the transepithelial electrical resistance to fall during the 40-min incubation period. To assess the variability in this response and the statistical difference between groups, the transepithelial electrical resistance measurements from six different experiments were compared (Table I). The mean starting resistance of the epithelial monolayers used for either the 5 or 10×10^6 neutrophils/ml experiments was 185.7 ± 22.7 (range, 266.3–121.6) and 197.2 ± 32.6 (range, 305.3–117.2), respectively. There was no statistical difference between their starting resistance ($P > 0.40$). As seen in Table I, a suspension of 5×10^6 neutrophils/ml stimulated to traverse the epithelium in response to 10^{-7} M fMLP caused no significant difference ($P > 0.475$) between the mean starting resistance and mean ending resistance after a 40-min incubation. In contrast, a concentration of 10×10^6 neutrophils/ml, under chemotactic conditions, caused an average fall in resistance of 45.7% (range, 31–65%) to 109 ohms·cm² ($P < 0.05$) by 40 min. When cross-sections of these experimental monolayers were examined by light microscopy (Fig. 4, *a* and *b*), the epithelium appeared intact.

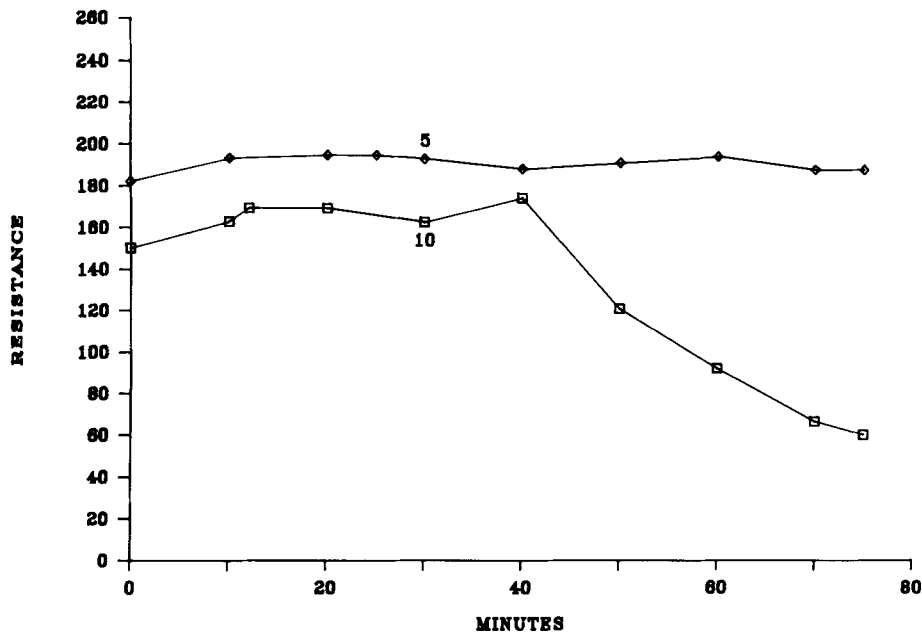


Figure 3. Effect of increasing neutrophil concentration on the trans-epithelial electrical resistance. Neutrophils at a concentration of 5×10^6 /ml (5) or 10×10^6 /ml (10) were added to the apical compartment at the time indicated by the numerals and stimulated to traverse the epithelial monolayer in response to 10^{-7} fMLP added to the basal compartment. Over the next 40 min the resistance of the monolayer exposed to 5×10^6 cells/ml remained stable while the resistance of the monolayer exposed to 10×10^6 cells/ml fell 61.5%.

Neutrophils that had traversed the epithelium were caught beneath the monolayer at the surface of the small pore size filter (0.45 μ m). Occasionally, areas of the epithelia exposed to 5×10^6 neutrophils/ml (Fig. 4 a) formed domes over large clusters of neutrophils that had emigrated beneath the monolayer. In contrast, all epithelia exposed to 10×10^6 neutrophils/ml (Fig. 4 b) had areas of dome formation over large accumulations of neutrophils.

The number of neutrophils that migrated across the filter and the number of invasion sites where neutrophils had traversed the monolayer were quantitated for both experimental groups (Table I). When the neutrophils were suspended at a concentration of 5×10^6 neutrophils/ml, a mean of 8.4 ± 2.5 neutrophils/mm epithelium traversed the epithelia. This was equivalent to the mean total emigration per monolayer of $\sim 54,000$ neutrophils. At concentrations of 10×10^6 neutrophils/ml, there was an eightfold increase in the average number of neutrophils/mm (66.0 ± 19.4 neu-

trophils/mm epithelium, $P < 0.01$). This resulted in an average total accumulation of 425,500 ($P < 0.01$) neutrophils that had traversed per monolayer. It is of interest that only 2.2% of the 5×10^6 neutrophils/ml and 8.5% of the 10×10^6 neutrophils/ml traversed the monolayer in 40 min. In addition, at the higher concentration of neutrophils (10×10^6 cells/ml) there was a fivefold increase in the number of invasion sites per millimeter of epithelium (10.6 ± 2.4 vs. 2.3 ± 0.6 ; $P < 0.005$) and a doubling in the number of neutrophils that traversed each invasion site (6.5 ± 1.1 vs. 3.5 ± 0.3 ; $P < 0.0125$).

Variation in Pore Size of the Filter

While the use of 0.45- μ m pore filters enables one to quantify both the number of emigrated neutrophils and the number of neutrophil invasion sites, it does result in a lifting of the epithelium over the cells that accumulate at the surface

Table I. Effect of Neutrophil Concentration on Epithelial Permeability

	5×10^6 PMNs/ml*	10×10^6 PMNs/ml
Starting resistance [‡]	185.7 ± 22.7 [§]	197.2 ± 32.6
Ending resistance	188.9 ± 20.8	109.3 ± 23.6
Percent change in resistance	$+2.6 \pm 2.8$	-45.7 ± 5.7 [¶]
No. of PMN invasion sites/mm epithelium	2.3 ± 0.6	10.6 ± 2.4 ^{**}
No. of PMNs traversed/mm epithelium	8.4 ± 2.5	66.0 ± 19.4 ^{††}
No. of PMNs traversed/PMN invasion site	3.5 ± 0.3	6.5 ± 1.1 ^{§§}
No. of PMNs traversed/monolayer	$53,965 \pm 16,191$	$425,506 \pm 124,713$ ^{††}
Percent of PMNs traversed/monolayer	2.2 ± 0.6	8.5 ± 2.5

* 0.5 ml of a suspension of neutrophils (PMNs) were placed above epithelial monolayers ($n = 6$) and stimulated to traverse the epithelium in response to 10^{-7} M fMLP for 40 ± 2 min.

[‡] In ohms \cdot cm².

[§] Mean \pm SEM.

Comparison between 5 and 10×10^6 PMNs/ml:

^{||} $P < 0.025$

[¶] $P < 0.0005$

^{**} $P < 0.005$

^{††} $P < 0.01$

^{§§} $P < 0.0125$

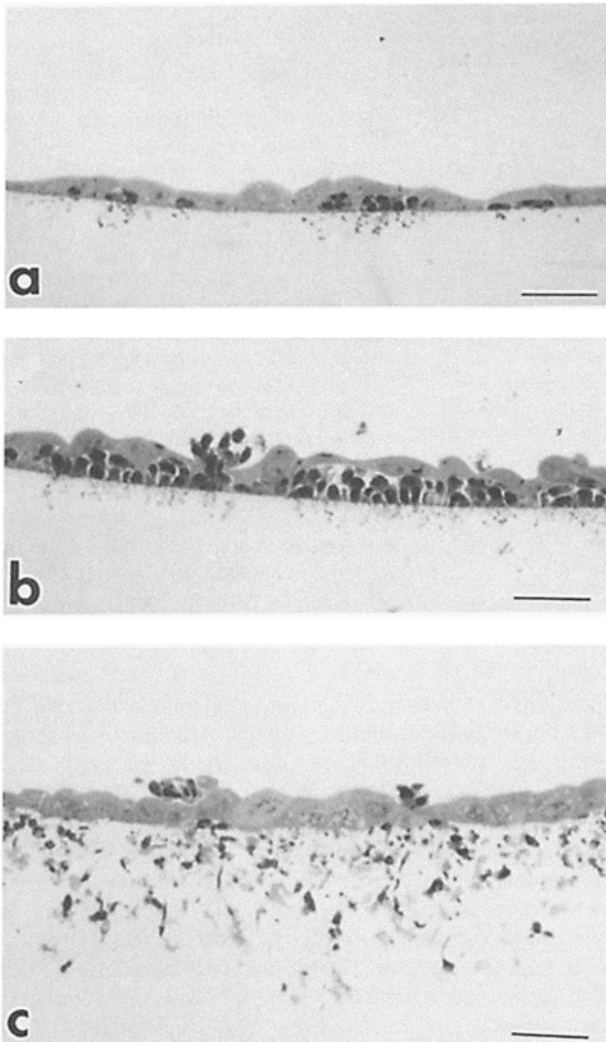


Figure 4. Light micrographs of neutrophil migration across epithelial monolayers in response to a gradient of 10^{-7} M fMLP for 45 min. (a) Neutrophils (5×10^6 cells/ml) have migrated across the epithelium and are trapped beneath the monolayer at the surface of the 0.45- μ m pore filter. (b) When the neutrophil concentration was doubled (10×10^6 cells/ml) greater numbers of neutrophils traversed the epithelium and were caught at the surface of the 0.45- μ m pore filter. The epithelium formed domes over these cells. (c) A larger pore size filter (3.0 μ m) permitted neutrophils (10×10^6 cells/ml) to continue their migration into the filter. This reduced the number of neutrophils that accumulated beneath the monolayer and the lifting of the epithelium. (a-c, toluidine blue; Bar, 31.1 μ m)

of the filter. To be sure the fall in resistance was not an artifact of the system, the design of the experiment was modified. Monolayers were grown on 3.0- μ m pore filters. This pore size enables neutrophils that have traversed the epithelium to continue their migration through the filter. When 10×10^6 neutrophils/ml were stimulated to migrate across these monolayers for 40 min, the epithelium remained intact and adherent to the filter surface (Fig. 4 c) but the transepithelial electrical resistance of the monolayers still fell. The reason for the fall in resistance was not morphologically apparent even at the ultrastructural level. The transepithelial electrical resistance measurements from five experiments in which 10×10^6 neutrophils/ml were stimulated to traverse

the epithelium grown on 3.0- μ m filters were normalized. The results were averaged and compared with the results obtained from five monolayers grown on 0.45- μ m pore filters under similar conditions (Fig. 5). There was no significant difference ($P > 0.1$) between the mean starting resistance of these two groups. The transepithelial electrical resistance of the monolayers grown on either filter fell similarly to an equivalent degree (41% on 3.0 μ m vs. 45% on 0.45 μ m; $P > 0.3$) after 10×10^6 neutrophils/ml were stimulated to traverse the epithelium for 40 min.

Recovery of Resistance

The process of directed migration of 10×10^6 neutrophils/ml across monolayers grown on 3.0- μ m pore filters for 40 min caused a fall in resistance that ranged between 27.7 and 56.2%. At this time the remaining neutrophils in the apical compartment and the chemoattractant in the lower compartment were removed. The monolayers were washed carefully in buffer and then incubated further in Gey's. Monolayers whose resistances had dropped $< \sim 45\%$ were able to totally recover their resistance between 30 and 60 min (Fig. 6). Those monolayers in which the resistance decreased $\geq 45\%$ recovered to within $23.5\% \pm 1.1\%$ (range, 21.2–26.1) of their original resistance in 30–60 min. The failure of these monolayers to completely recover did not appear to be due to the death of any of the epithelial cells. This was assessed by exclusion of trypan blue. Total recovery of the transepithelial electrical resistance of monolayers with falls $\geq 45\%$ may take longer than we measured.

Effect of Random Migration and Chemokinesis

The previous studies indicate that when 10×10^6 neutrophils/ml are stimulated to traverse the monolayer under conditions of chemotaxis, the transepithelial electrical resistance of the monolayer decreases with time. However, it is not clear whether the mere presence of a similar concentration of cells above the epithelium would have a similar effect. To examine this, monolayers were exposed to 5 or 10×10^6 neutrophils/ml under conditions of either random migration (Gey's in both compartments) or chemokinesis (10^{-7} M fMLP in both compartments; data not shown). Regardless of cell concentration or condition, less than one neutrophil per millimeter of epithelium traversed the monolayer. In addition, there was no significant decrease in resistance ($\sim 4\%$). Thus, the physical presence of neutrophils at the apical epithelial surface in the presence or absence of fMLP did not appear to influence the transepithelial electrical resistance. This is consistent with the results reported by Sugahara et al. (43).

Reversal of Neutrophil Migration

Neutrophils at a concentration of 5×10^6 cells/ml were able to migrate across the monolayer once without affecting the epithelial permeability. It was not clear whether the return migration of the same neutrophils across the same monolayer, and possibly the same junctions, would affect the permeability of the epithelium. Therefore, the transepithelial electrical resistance of an epithelium grown on a 0.45- μ m pore filter was continuously monitored while 5×10^6 neutrophils/ml first traversed the epithelium from the apical to basal direction (Fig. 7). After 30 min the neutrophils that had

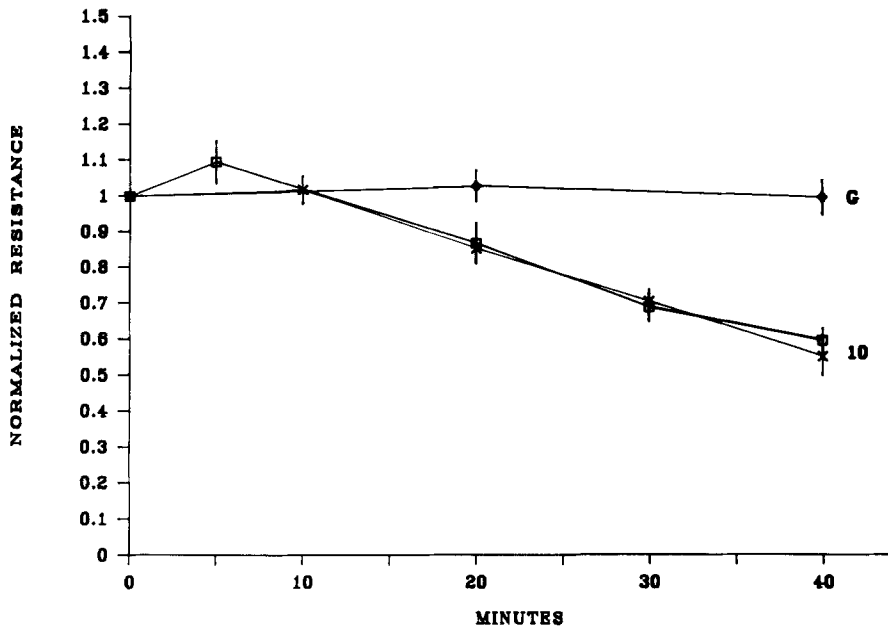


Figure 5. The effect of neutrophil migration (10×10^6 neutrophils/ml) across epithelial monolayers grown on 0.45- and 3.0- μm pore size filters. Neutrophils were stimulated to migrate across epithelial monolayers in response to 10^{-7} M fMLP for 40 min at 37°C . The normalized measurements with similar time points (± 2 min) from each experimental condition were averaged. The resistance decreased at a similar rate regardless of whether the emigrated neutrophils accumulated beneath the monolayer at the surface of the 0.45- μm pore filter (10, X, $n = 5$) or if they were able to migrate into the 3.0- μm (10, open box, $n = 5$) pore size filter. By 20 min the resistance had decreased significantly ($P < 0.05$) from the Gey's control (G, solid diamond, $n = 8$).

not migrated were removed and the chemotactic gradient was reversed. This stimulated the emigrated neutrophils to migrate (now in a basal to apical direction) back across the same area they had just traversed. This reversal resulted in a ~ 33 – 58% fall in resistance within 5 min. In general, the higher the starting resistance of the epithelium, the less the fall in resistance of the monolayer 5 min after reversal. After this sudden fall, the resistance of the monolayer gradually returned to its initial levels by ~ 55 min (Fig. 7). This decrease in resistance was not observed under control conditions when the chemotactic gradient across the monolayers was reversed in the absence of neutrophils.

To understand the relationship between the electrical measurements and the migratory process, filters were fixed at various times during the reversal process and examined by

either light microscopy or scanning electron microscopy. Before reversing the gradient, neutrophils could be observed beneath the monolayer at many locations, either in small clusters, in pairs, or singly. Within 5 min of reversing the chemotactic gradient, neutrophils could be observed beginning to migrate back across the monolayer (Fig. 8 *a*). Scanning electron microscopic examination of these cells revealed pseudopods emerging from the intercellular crevices between epithelial cells (Fig. 9 *a*). As remigration continued (15–30 min) those regions where many neutrophils had originally accumulated beneath the monolayer now contained groups of emerging neutrophils. When these cells reached the apical epithelial surface they formed clusters and adhered by fine tendrils to one another and to the newly traversing neutrophils (Figs. 8 *b* and 9 *b*). When the last neutrophil in

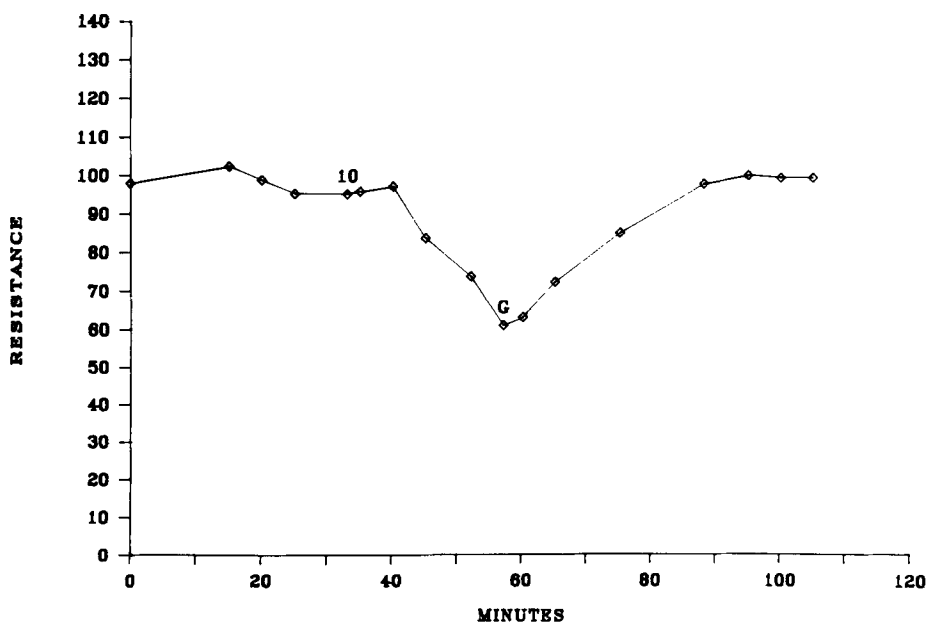


Figure 6. Recovery of the transepithelial electrical resistance after the migration of neutrophils at a concentration of 10×10^6 neutrophils/ml. The transepithelial electrical resistance of the epithelial monolayer was continuously monitored. After 35 min neutrophils were placed in the apical compartment (10) and were stimulated by 10^{-7} M fMLP to traverse the epithelium grown on 3.0- μm pore size filters for 22 min. The resistance of the monolayer decreased 35.2%. The cells that had not migrated and the chemoattractant were then removed. The monolayers were washed and allowed to incubate in Gey's (G). The resistance returned to its initial values within 31 min.

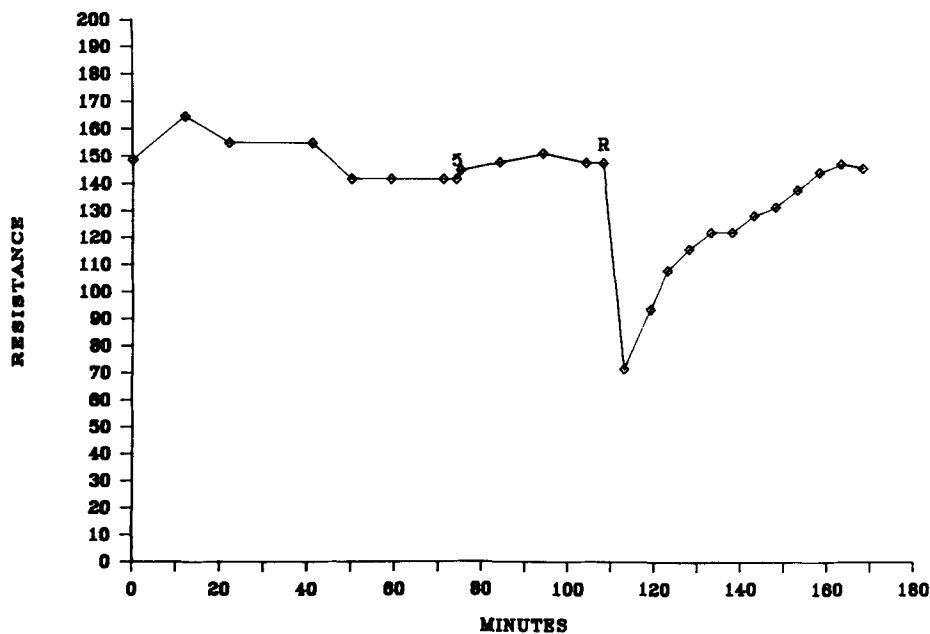


Figure 7. Continuous transepithelial electrical resistance studies during the migration and remigration of neutrophils at a concentration of 5×10^6 cells/ml. The transepithelial electrical resistance of the epithelial monolayer was continuously monitored at 37°C . After 65 min neutrophils were added to the apical compartment (5) and stimulated to migrate across the monolayer for 30 min. The resistance of the monolayer remained stable during this period. The chemotactic gradient was then reversed (R). This caused a rapid drop (51.6%) in resistance within 5 min. Over the next 55 min the resistance returned to its initial starting level.

the group completed its re-migration across the monolayer, the cluster of neutrophils detached and floated away. Thus, by 60 min after reversal (Fig. 8 c), when the resistance had returned to its initial level, the epithelial monolayer appeared intact and very few neutrophils remained either beneath the monolayer or attached to its apical surface.

Discussion

Through the use of an *in vitro* model it has been possible to eliminate serum and connective tissue factors and to examine the effect of the chemoattractant and the migratory leukocytes on the permeability of a renal epithelium. The synthetic peptide fMLP, which is equivalent to the major chemoattractant produced by bacteria (31), was used to stimulate neutrophil emigration across the epithelium. fMLP has been shown *in vivo* to affect the permeability of the intestinal epithelium (29). However, in the present study, neither the concentration of the chemoattractant nor its location above or below the monolayer elicited a change in the transepithelial electrical resistance of the renal epithelium. This confirms previous reports that fMLP has no effect on the permeability of blood vessels (23, 44) and endothelial (16) or MDCK monolayers (8).

The apical to basal migration of the lower concentration of neutrophils (5×10^6 cells/ml) did not increase the permeability of the epithelium, as the transepithelial electrical resistance of the monolayer remained stable throughout a 40-min migration period. This was true regardless of either the starting resistance of the monolayer (226–122 ohms-cm²) or when an average of eight neutrophils per millimeter of epithelium traversed the monolayer. The number of invasion sites per millimeter of epithelium varied with the starting resistance and ranged from one to five sites per millimeter of epithelium with an average of two and one-third sites per millimeter of epithelium. From a previous study (36), we know that these invasion sites are also impermeable to macromolecules. Since a basic function of epithelia is to form a

wall between two different environments, the ability of neutrophils to traverse an epithelium without disturbing this barrier is very important.

Relatively few of the added neutrophils actually migrate across the renal epithelium. Keller et al. (25) and Harvath and Leonard (17) have shown that in chemotactic studies only 15–40% of the available neutrophils respond to a gradient of fMLP and migrate into plain micropore filters. The renal epithelium appears to restrict the number of emigrating neutrophils even further (<10%). This may be due to the tightness of the epithelial occluding junctions and the need to have a population of neutrophils that are not only able to migrate but are able to open tight junctions.

In experiments in which the chemotactic gradient was reversed, the emigration of the lower concentration of neutrophils (5×10^6 cells/ml) was associated with a fall in the transepithelial electrical resistance. In this situation, the emigrated neutrophils were stimulated to migrate back across the same monolayer. Within 5 min the resistance fell ~48% and then returned to its initial levels as the neutrophils traversed the monolayer. The cause(s) for the sudden decrease in resistance is not clear, but may be due to synchronous opening of many junctional sites; the re-traversal across the same, possibly weakened, junctions; the direction (basal to apical) of migration; or a combination of these. Further studies are necessary to resolve this issue.

There was also a significant fall ($46 \pm 14\%$) in the transepithelial electrical resistance of the monolayer when the number of neutrophils migrating across the monolayer was increased by doubling the neutrophil concentration to 10×10^6 cells/ml. In fact, the more neutrophils that traversed the monolayer, the higher the percent decrease in resistance ($r = 0.98$; $P < 0.001$). At the higher concentration, an average of 66 neutrophils traversed the monolayer per millimeter of epithelium at an average of 10.6 sites per millimeter of epithelium. The presence of 10×10^6 neutrophils/ml under conditions of either random migration or chemokinesis caused little to no transepithelial migration of neutrophils and had

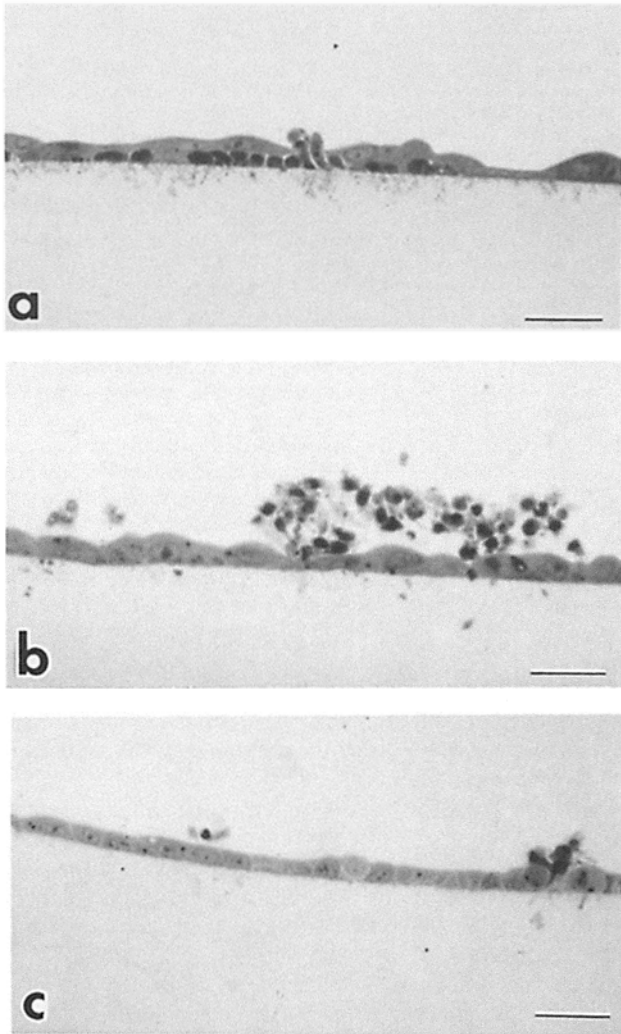


Figure 8. Light micrographs of the process of remigration of neutrophils at a concentration of 5×10^6 cells/ml. (a) 5 min after reversing the chemotactic gradient neutrophils can be seen emerging from beneath the monolayer. (b) By 30 min most of the neutrophils have crossed the monolayer and are present in clusters at the apical epithelial surface. (c) After 60 min only a few neutrophils remain associated with the monolayer. The monolayer itself appears intact. (a–c, toluidine blue; Bar, 32.2 μ m).

no affect on epithelial permeability. This suggests that it is the process of migration of many neutrophils across the monolayer rather than just their presence above the monolayer that causes the fall in resistance.

The decrease in resistance caused by the migration of 10×10^6 neutrophils/ml was totally reversible within ~ 1 h if the transepithelial electrical resistance was initially lowered $<45\%$. A decrease in resistance of $>45\%$ delayed the recovery period such that monolayers only recovered an average of 23% of their initial resistance by 60 min. A similar variability in recovery of resistance occurred in MDCK monolayers after removal of extracellular calcium (4, 14, 32). In these experiments the recovery period appeared to be dependent upon the length of time (>20 min) the junctions were kept open rather than the percent fall in resistance. In our studies the time period for neutrophil migration across the monolayer was always 40 min, although the length of time

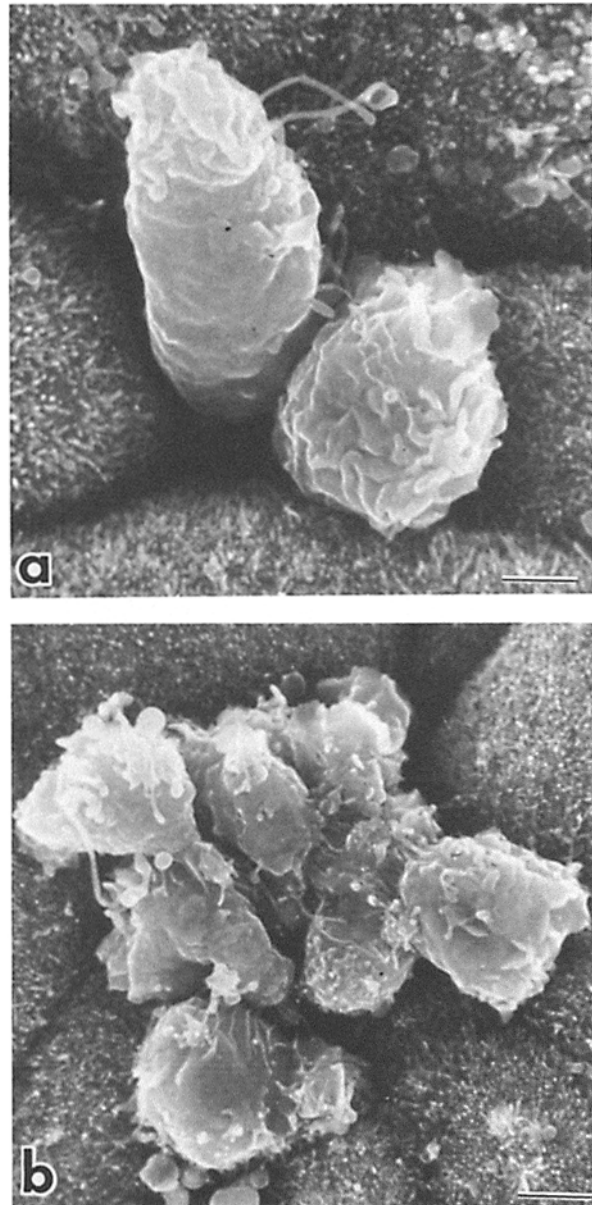


Figure 9. Scanning electron micrographs of neutrophils in the process of remigrating across the monolayer. (a) At 5 min neutrophils are emerging from beneath the monolayer. Bar, 2.5 μ m. (b) By 15 min large clusters of neutrophils are seen at the apical surface adhering to each other with thin tendrils. Bar, 2.9 μ m.

each junction was kept open probably varied from one invasion site to another. Falls in resistance $>45\%$ may reflect monolayers in which a number of invasion sites were kept open for longer lengths of time, thereby requiring longer time to repair.

Both Griep et al. (13) and Gonzalez-Mariscal et al. (12) found that in established MDCK monolayers de novo protein synthesis was not required for the resealing of junctions after calcium chelation. In our studies, the rapid recovery of monolayers whose resistance fell $<45\%$ also suggests that protein synthesis is not required for junctional repair after neutrophil emigration. Whether protein synthesis is required after falls in resistance of $>45\%$ remains to be determined.

Recently, Evans and co-workers examined the migration of

rat peritoneal exudate cells across a low and high resistance strain of kidney MDCK epithelial cells (8). These cells (62% neutrophils, 34% macrophages, and 4% lymphocytes), which were collected in response to 1% oyster glycogen, appeared to have a more disruptive effect on the integrity of the kidney epithelium than human peripheral blood neutrophils. The emigration of peritoneal exudate cells decreased the transepithelial electrical resistance of the kidney epithelium at least 30% even with low migratory cell concentrations (4.1×10^6). The repair of these monolayers required at least 20 h to re-establish the initial transepithelial electrical resistance. The cause for the different reaction to the migration of human peripheral blood neutrophils and rat peritoneal exudate cells may be due to the presence of macrophages and lymphocytes in the exudate, species differences, or the amount of cell activation.

Through the use of an in vitro model system, it is possible to determine the factors that affect the permeability of an epithelium during an inflammatory response. By eliminating connective tissue and serum factors, the direct effect of neutrophil migration on the permeability of an epithelium was assessed. The results of this study indicate that neutrophil migration can occur with or without an increase in epithelial permeability. The fall in transepithelial electrical resistance that sometimes accompanies neutrophil emigration does not appear to result from destruction of the epithelium, but rather from a temporary loosening of the occluding junctions.

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