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The Effect of Neutrophil Migration and Prolonged Neutrophil Contact on Epithelial Permeability

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The effect of neutrophil migration and prolonged neutrophil contact on epithelial permeability was examined. Although neutrophil migration was not associated with a change in epithelial permeability, prolonged neutrophil–epithelial contact following migration resulted in an increase in epithelial permeability. These results were not altered by catalase, a specific neutrophil elastase inhibitor, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone or cyclohexamide. This suggests that neutrophil migration does not occur via an H_2O_2-induced reversible mechanism of junctional opening, which we describe herein. (Am J Pathol 1987, 129:302–312)

THE EXTRAVASCULAR accumulation of neutrophils is a recognized hallmark of the acute inflammatory response. It appears that in the presence of a variety of chemotactic stimuli neutrophils will adhere to and traverse vascular endothelium.[1,11] Ultrastructural studies indicate that this migration occurs via intercellular junctions without apparent injury to endothelial cells.[12,15,16] In many situations the neutrophils then migrate through an epithelium. Again the neutrophils appear to move via intercellular junctions,[21] and the migration is not necessarily associated with alterations in epithelial permeability.[22] This suggests that the process of neutrophil accumulation at an inflammatory site can occur without cellular injury or increased epithelial permeability.

However, a second hallmark of the inflammatory response is an increase in endothelial and epithelial permeability, and the point at which this occurs is not clear. There is strong evidence that activated neutrophils can affect epithelial permeability and that neutrophil products such as oxidants and proteases will affect the permeability of both cultured epithelial monolayers and isolated perfused organs.[13,17,30,31] It appears to us that although neutrophil migration per se may not increase epithelial permeability, subsequent neutrophil–epithelial interactions could result in an alteration of permeability.

Accordingly, we carried out experiments to investigate the mechanism of neutrophil migration through a cultured epithelial monolayer and to study the effect of prolonged contact between activated neutrophils and the epithelium before and after migration.

This work has been previously published in abstract form.[26,27]

Materials and Methods

Cell Culture

Madin–Darby canine kidney (MDCK) cells (American Type Culture Collection) were chosen as a prototype epithelium because they are well characterized and have been documented to form tight intercellular junctions in culture.[3] The cells were maintained in culture through serial passages in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 5% lactoalbumin hydrolysate, and 100 U/ml penicillin. The cells were harvested with 2.5% trypsin in Hanks' balanced salt solution (HBSS) without calcium or magnesium.

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They were plated at a density of approximately 1–2 × 10^6 cells per milliliter on either 0.45-μ or 5-μ pore size micropore filters (HAMK, Millipore). The filters had been previously coated with rat tail collagen, fixed with gluteraldehyde, and extensively washed.[24] The cells were cultured for 6–14 days, of which time they were confluent monolayers with no evidence of cell death as determined by Trypan blue exclusion. By light and electron microscopy there was no evidence that the cells were forming multiple layers. All tissue culture materials were from Costar (Cambridge, Mass).

Alveolar Type II cells were isolated from adult Sprague–Dawley male rats by tissue dissociation with elastase and partial purification on a metrizamide density gradient.[5] The cells were suspended at 2 × 10^6 cells/ml in DMEM supplemented with 10% porcine serum, 0.2 μM dexamethasone, 2 mM glutamine, 10 μg/ml gentamicin, 100 units/ml penicillin, and 50 μg/ml streptomycin and were plated on rat tail collagen-coated Millipore filters. The cells cultured on filters for 6–14 days form a morphologically and pharmacologically polarized epithelium.[18,32]

Neutrophil Isolation

Neutrophils were isolated from the citrated venous blood of normal, healthy volunteers by the method of Haslett et al.[8] The neutrophil fraction was isolated by discontinuous plasma Percoll gradients using lipopolysaccharide (LPS)-free reagents. The neutrophil fraction was 95% pure and 98% viable by Trypan blue exclusion.

Preparation of Opsonized Zymosan (OPZ)

Zymosan A (Sigma Co., St. Louis, Mo) was boiled, washed, and incubated with a pooled human serum pool for 30 minutes at 37 C. The zymosan was then washed and resuspended in saline.

Electrophysiology

Measurements of electrical resistance across the monolayers were made in two different systems. The first system utilized a modified Ussing chamber with the monolayer oriented vertically between two halves of the chamber. The monolayers were bathed in HBSS which had been supplemented with 0.1% bovine serum albumin, 20 mM HEPES, and 20 mM $NaHCO_3$ (HBSA). The solution volume was 15 ml in each half of the chamber, and it was circulated by bubbling with 95% air and 5% CO_2 (pH 7.4). The chamber was maintained at 37 C.

The transepithelial potential difference was measured via 3M KCl/agar bridges, which connected the bathing solution to a Dual Voltage Clamp Apparatus (University of Iowa Bioengineering Department) via two calomel electrodes. Resistance was calculated by intermittently passing a 1-second 10 μ ampere bipolar current pulse across the monolayer via NaCl/agar bridges, recording the change in voltage, and applying Ohm's law. Transepithelial resistances of our MDCK monolayers ranged from 110 to 1150 ohms/sq cm.

The second system utilized another modified Ussing chamber in which the monolayer was oriented horizontally between the two halves of the changer. This chamber had a volume of 3 ml per side and was maintained at 37 C, but the solutions were not circulated. The bioelectric properties were measured exactly as described for the vertical system.

Chemotaxis

All neutrophil chemotaxis was performed in the horizontal chamber. The two halves of the chamber were divided by either an epithelial monolayer on a collagen-coated 5-μ-pore filter with an underlying uncoated 0.45-μ-pore catch filter or by an epithelial monolayer on a collagen-coated 0.45-μ-pore filter. The neutrophils were added to the top compartment at a final concentration of 3 × 10^6/ml in HBSA. Formyl-methyl-leucine-phenylalanine (FMLP) 10^{-8} M in HBSA served as a chemoattractant in the lower well. Electrical resistance was monitored as described above.

At the completion of each experiment the filters were removed from the chamber, immediately fixed with 20% butanol in ethanol, stained with Weigart's stain, dehydrated with ethanol and butanol, then cleared with xylene. Neutrophil migration was quantitated by light microscopy.

Hydrogen Peroxide Assay

Hydrogen peroxide release from neutrophils was determined by means of the scopoletin oxidation assay.[29] In the presence of horseradish peroxidase scopoletin oxidizes and loses fluorescence at a rate proportional to the concentration of hydrogen peroxide in the medium. Neutrophils (PMNs) (3 × 10^6/ml) and opsonized zymosan (OPZ) (4–5 particles/PMN) were added to scopoletin (10 μM), horseradish peroxidase (200 μg/ml), and Na azide (1 mM) in a glass curette. Fluorescence was measured on a spectrofluorimeter (Perkin-Elmer) with the activating light set at a 350-nm wavelength and the intensity of the emission monitored at 460 nm.

Elastase Assay

^{3}H-elastin was prepared using a modification of the NaBH4 reduction procedure.[34] PMNs and OPZ particles were incubated at 37 C with 200 μg of ^{3}H-elastin in 100 mM Tris buffer with 5 mM $CaCl_2$ 0.02% NaN_3 (pH 7.8). The samples were then centrifuged for 3 minutes in a Beckman microfuge, and radioactivity in the supernatant was measured by liquid scintillation spectrometry. One unit of elastase activity was defined as the amount required to solubilize 1 μg of elastin in 1 hour at 37 C.

Viability

Acridine orange and ethidium bromide (Sigma) were each added to PBS to a final concentration of 10^{-5} mg/ml. Two milliliters of this solution was placed in a 35-mm tissue culture disk containing a cell monolayer on a filter for 1 minute. The monolayers were then examined by means of an inverted fluorescent microscope (Ultrastar), and the ratio of viable (green) cells versus noviable (orange) cells was determined.[25]

Protein Synthesis Inhibition

MDCK cells grown on plastic in 25-mm wells were incubated with cycloheximide (0.25 μg/ml, 2.5 μg/ml, and 25 μg/ml) for 2 hours. The monolayers were then washed in leucine-free RPMI medium, and the appropriate concentrations of cycloheximide were again added to the wells. Fifty microcuries of ^{3}H-leucine was added to each well. The cells were incubated at 37 C in 7% CO_2 for 1 hour, then washed in cold PBS, scraped into PBS with 5% bovine serum albumin, and precipitated with TCA. The precipitates were suspended in 0.1 M NaOH and diluted 1 : 10 in scintillation fluid, and cell-associated radioactivity was determined on a Beckman scintillation counter.

Avidin–Biotin Immunofluorescence Techniques

MDCK cells were plated on 0.45-μ and 5-μ Millipore filters that were coated with 100 μg/ml human fibronectin (Collaborative Research, Inc. Waltham, Mass). After the measurement of transepithelial resistance during H_2O_2 exposure or neutrophil migration, the monolayers were fixed with 2% paraformaldehyde. Nonspecific absorption was blocked with 20 mg/ml human serum albumin (Calbiochem, Behring Diagnostics, La Jolla, Calif) and 200 μg/ml Avidin D (Vector Laboratories, Burlington, Calif). The monolayers were then incubated with sheep anti-fibronectin (kindly provided by Dr. R. A. F. Clark[4] at 37 C for 1 hour, washed, and then incubated with biotinylated antisheep IgG (5 mg/ml, Vector) at 37 C for 1 hour. The monolayers were washed again and incubated with fluorescein isothiocyanate–avidin (20 μg/ml, Vector) for 1 hour. The monolayers were mounted on slides in 1 : 1 glycerol : PBS.

Materials

FMLP was purchased from Vega Biochemicals (Tucson, Ariz). Catalase (from bovine liver, 11800 U/mg) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, Mo). Purified pancreatic elastase, 34.50 μg/ml, was purchased from Elastin Products Corporation (St. Louis, Mo). Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (AAPVCK) was purchased from Bachem Inc. (Torrance, Calif).

Results

To test the hypothesis that neutrophils can accumulate at a site of inflammation without altering epithelial permeability, the effect of neutrophil activation and subsequent migration of neutrophils through an epithelial monolayer was examined. Initially, MDCK cell monolayers grown on 5-μ pore size rat tail collagen-coated filters were mounted horizontally between two halves of a modified Ussing chamber. The two halves of the chamber were filled with HBSA. The electrical resistance was measured continuously for 30 minutes while the monolayers equilibrated. Neutrophils (3×10^6/ml) and opsonized zymosan (4–5 particles per PMN) were then added to the upper chamber and the transepithelial electrical resistance was monitored for 120 minutes. No change in electrical resistance was seen (data not shown), and there were no neutrophils adhering to the epithelial monolayer when the filters were fixed, stained, and examined with light microscopy.

The process of transepithelial migration was subsequently examined. MDCK cell monolayers grown on 5-μ pore size rat tail collagen-coated filters were mounted horizontally with a 0.45-μ pore size uncoated filter between two halves of a modified Ussing chamber. The bottom half of the chamber contained FMLP 10^{-8} M in HBSA, and the top half contained HBSA alone. Again, the electrical resistance was measured continually for 30 minutes while the monolayers equilibrated. Neutrophils (3×10^6/ml) were then added to the top chamber, and resistance was monitored for another sixty minutes. Twenty-five ± 10 neutrophils per 400× field were counted on the

0.45-μ pore size catch filters at the end of the 60 minutes. As can be seen in Figure 1, there was no change in electrical resistance during the course of this neutrophil migration. This confirmed the investigations of Milks and Cramer, who demonstrated that neutrophil migration through an epithelial monolayer can occur without altering transepithelial resistance. Their studies demonstrated that the neutrophils migrate via intercellular junctions.[22]

Acute inflammation is often associated with altered epithelial permeability. Having shown that neutrophil migration through an epithelium may occur without altering its permeability, we hypothesized that prolonged neutrophil-epithelial contact following migration could alter epithelial permeability. To test this, MDCK monolayers grown on 0.45 μ rat tail collagen-coated filters (through which PMN cannot migrate) were mounted in the chamber, and the experiments were repeated using the above protocol. As can also be seen in Figure 1, there was a significant decrease in transepithelial resistance to 71.0 ± 7.9% in 60 minutes when neutrophils migrated through the epithelium and were subsequently trapped between the monolayer and the filter. The exact number of PMNs that migrated through the monolayer could not be quantitated, because it was impossible to always accurately determine which PMNs were attached to the monolayer and which had migrated beneath it. Even serial cut sections of the monolayer revealed only isolated scattered cells that had migrated. There were no areas where large numbers of PMNs had accumulated below the monolayer and no evidence of dome formation by the epithelial cells. In each case, however, the total number of PMNs either on or beneath the monolayer was less than 5 cells per HPF. This suggested to us that prolonged contact of activated neutrophils with the alveolar epithelium could cause increased permeability.

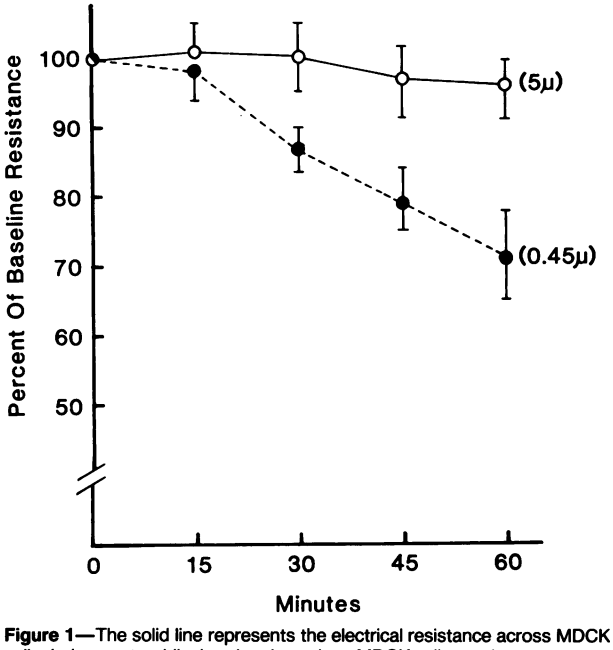

Figure 1—The solid line represents the electrical resistance across MDCK cells during neutrophil migration through an MDCK cell monolayer grown on a Millipore filter with an average pore size of 5 μ (n = 7). Resistance measurements ranged from 110 to 420 ohms/sq cm. The *dotted line* represents serial measurements of transepithelial resistance during neutrophil migration through MDCK cell monolayers grown on a Millipore filter with an average pore size of 0.45 μ (n = 7). Resistance measurements ranged from 140 to 480 ohms/sq cm.

For neutrophils to have migrated through epithelia via intercellular junctions without altering transepithelial electrical resistance, there had to have been a reversible mechanism of junctional opening and resealing induced either by the neutrophil or by the epithelium in response to the presence of the neutrophil. We speculated that neutrophils could release either oxidants and/or proteases in response to chemotactic stimuli. In low concentrations, these oxidants or proteases could result in a reversible opening of epithelial junctions; whereas in greater concentrations (such as could develop when the neutrophil was trapped between an epithelial monolayer and a 0.45-μ pore size filter), they could cause epithelial damage.

In our system 3×10^6 PMNs/ml activated by 4–5 OPZ particles per PMN produced 10^{-6} M hydrogen peroxide (scopoletin reduction) and 82.9 μ/ml of elastase (tritiated elastin assay). We then investigated whether these agents alone could affect epithelial resistance.

Purified pancreatic elastase (129.6 μ/mg) was added to the apical surface of MDCK monolayers grown on 0.45-μ pore size rat tail collagen-coated filters and mounted vertically in the Ussing chamber. Transepithelial resistance was monitored continually for 1 hour after the addition of the elastase in concentrations from 0.25 mg to 4 mg/ml, and no decrease was noted in transepithelial resistance (data not shown). The effect of H_2O_2 on the monolayers was then determined. MDCK cells on 0.45 μ filters were again mounted vertically between two halves of an Ussing chamber, allowed to equilibrate for 30 minutes, and H_2O_2 was added to the apical surface of the monolayers in doses of 10^{-8} M to 10^{-4} M. Each monolayer was exposed to a single dose of H_2O_2. Resistance was continually measured for 1 hour. H_2O_2 at 10^{-6} resulted in a decrease in resistance in 30% of the monolayers, but 10^{-5} M H_2O_2 always resulted in a drop in the resistance to zero (Figure 2). To determine that this change in transepithelial resistance was the direct effect of H_2O_2, an excess of catalase (2500 U/ml) which would be required to neutralize the H_2O_2 was added bilaterally in the chamber prior to the

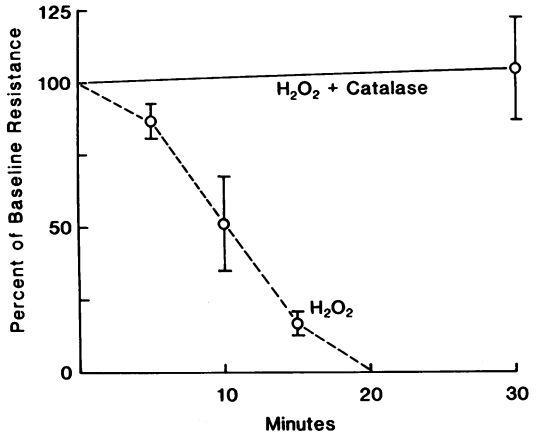

Figure 2—H_2O_2 (10^{-5} M) added at time 0 consistently resulted in a drop in resistance from baseline to 0 in 20 minutes (*dashed line*) (n = 8). Initial resistance of the monolayers, 160–1100 ohms/sq cm. The addition of catalase (2500 μ/ml) simultaneously with H_2O_2 (10^{-5} M) prevented the change in resistance (*solid line*) (n = 6). Initial resistance of the monolayers, 150–760 ohms/sq cm.

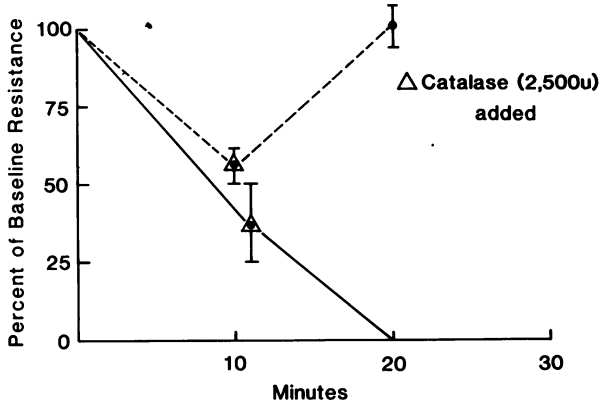

Figure 3—H_2O_2 (10^{-5} M) added to MDCK cells in an Ussing chamber with the subsequent addition of catalase (2500 μ/ml). Catalase added when resistance had fallen to 50% of baseline did not prevent resistance from falling to 0 (*solid line*) (n = 7). Initial monolayer resistance, 160–410 ohms/sq cm. Catalase was added when resistance had fallen to 50–60% of baseline resulted in the resistance returning to baseline (*dashed line*) (n = 5). Initial monolayer resistance, 280–1150 ohms/sq cm. The *bars* represent standard error.

addition of H_2O_2 (10^{-5} M). There was subsequently no decrease in resistance associated with the addition of H_2O_2 (Figure 2).

The data suggested that the alteration in permeability caused by H_2O_2 might be the result of the "opening" of intercellular junctions. We hypothesized that if H_2O_2 was responsible for the opening of junctions during neutrophil migration there might be a mechanism of reversibility. In order to determine whether the effects of H_2O_2 were reversible, catalase was added at varying time points following the addition of H_2O_2. MDCK monolayers on 0.45 μ filters were mounted vertically in the Ussing chamber, and transepithelial resistance was allowed to equilibrate. H_2O_2 (10^{-5} M) was added to the apical surface, and the resistance was continually monitored. Catalase (2500 μ) was added bilaterally when the transepithelial resistance had fallen either to less than 50% of baseline or to 50–70% of baseline. As can be seen in Figure 3, if the resistance had fallen to less than 50% of baseline in the presence of H_2O_2 (10^{-5} M), the subsequent addition of catalase did not prevent the resistance from continuing to fall to zero. However, if catalase was added when the resistance had fallen only to 50–75% of baseline, the resistance returned to baseline within 15 minutes. Thus, early alterations in epithelial permeability secondary to H_2O_2 were reversible.

Other investigators have shown that EGTA-induced calcium depletion caused an increase in the permeability of MDCK monolayers.[18] This process was reversible upon the readdition of Ca^{+2}, but it took 1–3 hours to return to baseline. This resealing process was not dependent on protein synthesis, but did require microfilament integrity.[18,20]

To determine whether the H_2O_2-induced permeability changes were similar to those produced by Ca^{+2} depletion, we examined the effect of cycloheximide on the process. Using ^{3}H-leucine incorporation to assay for protein synthesis, a 2-hour incubation with 25 μg/ml cycloheximide was shown to result in a 95% inhibition of the protein synthesis of MDCK cells. With 2.5 μg/ml cycloheximide, 89% of protein synthesis was inhibited. Accordingly, MDCK monolayers were mounted in the Ussing chamber, and cycloheximide (either 2.5 or 25 μg/ml) was added bilaterally. Resistance was monitored continually for 3 hours, and no change in resistance was noted. Having determined that 25 μg/ml cycloheximide did not affect MDCK cell monolayer integrity, the effect of H_2O_2 and catalase on monolayers incubated with 25 μg/ml cycloheximide was evaluated. Monolayers were placed in the chamber with cycloheximide 25 μg/ml added bilaterally. Resistance was monitored for 2 hours and remained stable. H_2O_2 10^{-5} M was then added to the apical surface and the resistance rapidly decreased to zero exactly as had been seen previously in the absence of cycloheximide (data not shown). In other monolayers catalase was added bilaterally when the resistance had fallen to 50–75% of baseline but it did not prevent the resistance from continuing to decrease toward zero (Figure 4). Thus, the reversible phase of H_2O_2-induced increased permeability of MDCK monolayers appeared to be, at least in part, dependent on protein synthesis.

To determine whether neutrophil-derived H_2O_2 was involved in neutrophil migration or the change in permeability associated with prolonged neutrophil–epithelial contact, the migration experiments were re-

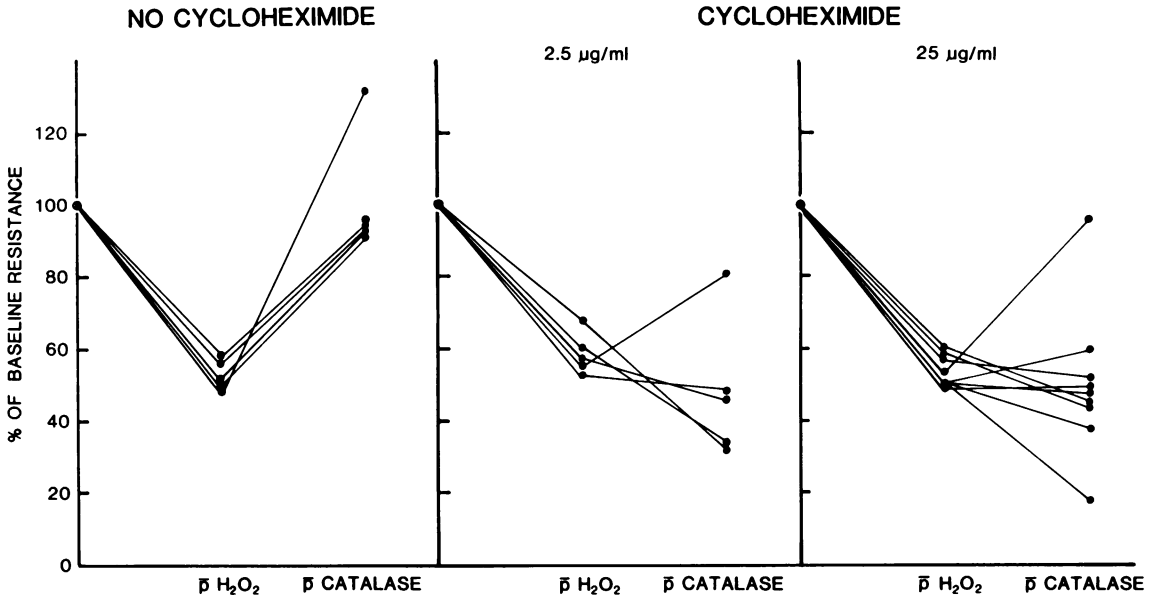

Figure 4—The first panel shows that an H_2O_2-induced decrease in resistance can be reversed if catalase is added before the resistance falls to <50% at baseline. The reversibility is inhibited by the presence of cycloheximide (2.5 and 25 μg/ml), as shown in the second and third panels. The initial resistance of the monolayers, 110–750 ohms/sq cm. H_2O_2 was added when the resistance was at 100% of baseline. Catalase was added when the resistance had fallen to 50–60% of baseline, which occurred within 10 minutes (labeled *p̄ H_2O_2* on the x axis). In the absence of cycloheximide resistance returned to baseline within 20 minutes and remained stable for at least 1 hour. In the presence of cycloheximide resistance did not return to baseline but was monitored until it had stabilized for 1 hour at which point a final resistance was determined (labeled *p̄ catalase* on the x axis).

peated in the presence of catalase. MDCK cells grown on 5-μ pore size filters were mounted horizontally in the Ussing chamber. FMLP 10^{-8} M with 2500 U/ml catalase was placed in the bottom compartment and 3×10^{-6} PMN/ml with 2500 U/ml catalase were placed in the top compartment. Transepithelial resistance was monitored for 1 hour, and neutrophil chemotaxis was quantitated at the end of the experiment. There was no change in electrical resistance from baseline, and there was no quantitative change in neutrophil chemotaxis from that which occurred in the absence of catalase (Figure 5, Table 1).

In light of recent data from other investigators[7,32] which showed that neutrophil disruption of a cellular monolayer was the result of neutrophil proteases, rather than oxygen radicals, we examined the affect of a neutrophil elastase inhibitor, AAPVCK. The addition of 10^{-4} M AAPVCK bilaterally did not result in any change in resistance across the MDCK monolayers on 5-μ-pore filters during neutrophil migration, did not quantitatively effect neutrophil migration, and did not prevent the decrease in resistance seen with neutrophil migration through monolayers on 0.45-μ pore size filters (Figure 6, Table 1). This concentration of AAPVCK had been shown to block neutrophil migration through elastin barrier.[6]

Thus, neutrophil migration was not impeded by the presence of catalase or AAPVCK. This suggested that the epithelial junctional opening and resealing which occurred during neutrophil migration was mechanistically different from the H_2O_2-induced junctional opening with catalase facilitated resealing. However,

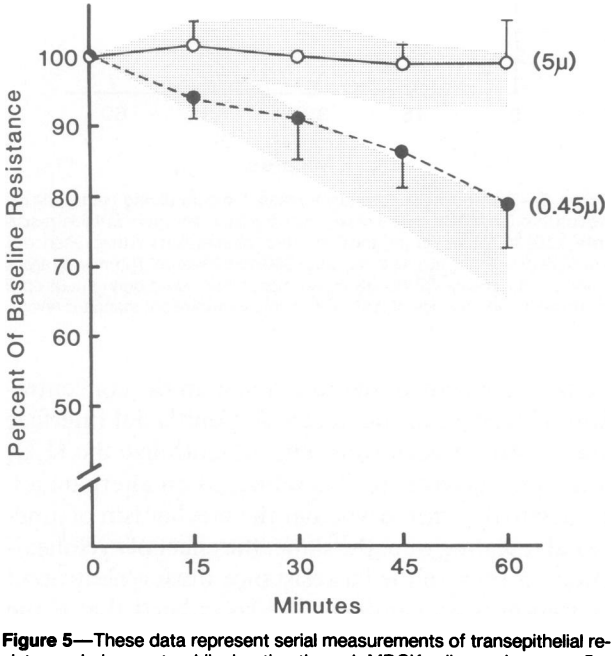

Figure 5—These data represent serial measurements of transepithelial resistance during neutrophil migration through MDCK cell monolayers on 5-μ pore size filter (*solid line*) (n = 5) (initial resistance, 150–760 ohms/sq cm) and 0.45-μ pore size filters (*dotted line*) (n = 7) (initial resistance, 110–680 ohms/sq cm) in the presence of catalase (2500 μ/ml). The shaded areas are the mean ± 2 SD of the resistance measured during neutrophil migration in the absence of catalase. The *bars* represent standard errors.

Table 1—Quantitation of Neutrophil Migration*

	No. PMNs per high-power field			
Catalase	29 ± 6 (700)	25 ± 5 (320)	7 ± 2 (160)	7.5 ± 2.5 (170)
Control	30 ± 9 (140)	30 ± 9 (140)	8 ± 3 (150)	8 ± 3 (150)
AAPVCK	27 ± 4 (240)	30 ± 6 (200)	15 ± 3 (150)	
Control	30 ± 5 (280)	30 ± 3 (230)	14 ± 4 (310)	
Cycloheximide	22 ± 8 (260)	14.5 ± 3.5 (220)	28 ± 6 (260)	
Control	18 ± 4 (180)	18 ± 4 (190)	26 ± 5 (250)	

*Expressed as mean number of neutrophils (± SD/high-power [5 fields counted] field) on 0.45-μ catch filter. Number in parenthesis is the resistance (ohms/sq cm) of the monolayer. Control numbers were derived from neutrophils migrating in response to FMLP through an epithelial monolayer in the presence of HBSA alone. The pairs of control and investigational migration studies were run simultaneously. Each column represents the results of a single experiment.

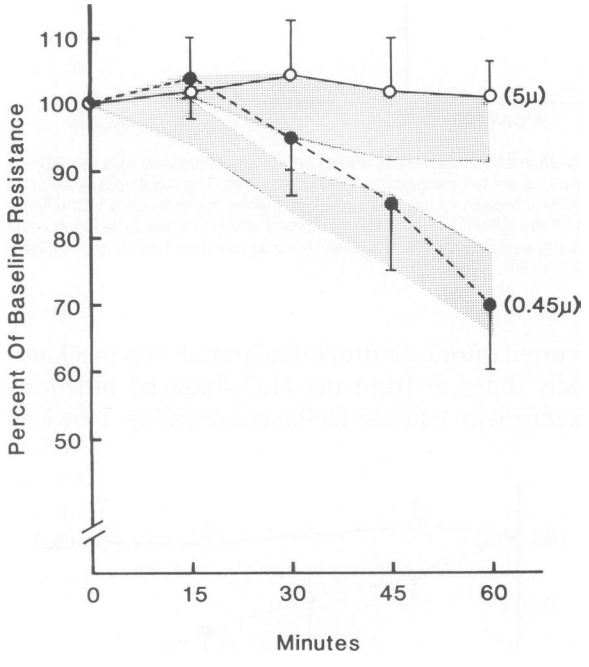

Figure 6—Transepithelial resistance measurements during neutrophil migration through MDCK cell monolayers on 5-μ (*solid line*) (n = 5) (initial resistance, 110–280 ohms/sq cm) and 0.45-μ (*dotted line*) filters in the presence of AAPVCK (n = 6) (initial resistance, 250–380 ohms/sq cm). The *shaded areas* represent the mean ± 2 SD of the resistance measured during neutrophil migration in the absence of AAPVCK. The *bars* represent standard errors.

we were concerned that in our system the concentration of catalase at the neutrophil/epithelial interface may not have been sufficient to neutralize the H_2O_2 being produced there. Therefore, as an alternate approach to determine whether the mechanism of junctional resealing were the same, the effect of cycloheximide on transepithelial resistance during neutrophil migration was examined. We speculated that if the maintenance of the epithelial resistance during neutrophil migration was protein synthesis-dependent, then the electrical resistance would fall during neutrophil migration through an MDCK monolayer on a 5-μ filter in the presence of cycloheximide. MDCK monolayers on 5-μ filters were mounted horizontally in the chamber. FMLP 10^{-8} was placed in the bottom compartment and cycloheximide 25 μg/ml was added bilaterally. Resistance was continually monitored for 2 hours and remained stable. Neutrophils, 3×10^{-6}/ml, were then added to the upper compartment, and the resistance was monitored for 1 hour. There was no decrease in electrical resistance. Quantitation of neutrophil migration revealed that there was no inhibition of migration in the presence of cycloheximide (Figure 7, Table 1).

To ascertain that our data on neutrophil migration was pertinent to epithelia other than MDCK cells, we repeated the initial migration studies with rat alveolar Type II cells. Neutrophil migration through type II cells monolayers on 5-μ pore size filters did not alter

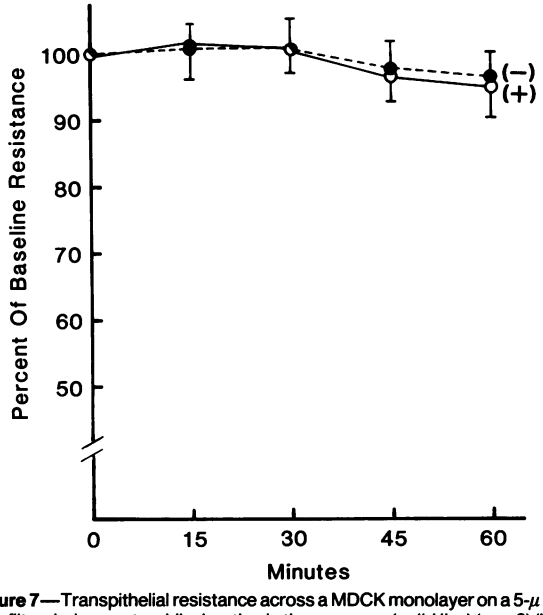

Figure 7—Transpithelial resistance across a MDCK monolayer on a 5-μ pore size filter during neutrophil migration in the presence (*solid line*) (n = 6) (initial resistance, 220–540 ohms/sq cm) and absence (*dotted line*) of cycloheximide, 2.5 μg/ml (n = 7) (initial resistance, 110–420 ohms/sq cm).

electrical resistance. However, as with MDCK cells, neutrophil migration through a Type II cell monolayers on 0.45 μ filters decreased the resistance to 71% ± 8% of baseline after 1 hour (data not shown).

It was assumed that the changes in resistance that we observed were the result of junctional alterations but to be certain that cell death was not a factor we stained the monolayers with acridine orange and ethidium bromide and observed the pattern of fluorescence after H_2O_2, cycloheximide, and neutrophil migration. Using this system viable cells exhibit a green fluorescence and dead cells an orange fluorescence. Untreated monolayers routinely fluoresced green, with no visible orange coloration. As a positive control, cells were shown to stain orange after freeze/thawing and mechanical trauma. However, there was no evidence of cell death after the resistance had fallen to zero in the presence of H_2O_2 (10^{-5} M immediately and up to 6 hours after the resistance had fallen), 2 hours after the addition of cycloheximide 25 μg/ml, or after neutrophil migration through MDCK monolayers on 5-μ or 0.45-μ pore size filters. There was also no visible evidence of disruption of the MDCK monolayers under any of those conditions. To further demonstrate that monolayer integrity was maintained throughout the experiments MDCK cells were plated on fibronectin-coated filters and exposed to antifibronectin antibodies after either neutrophil migration or exposure to H_2O_2 with a fall in resistance to zero. Penetration of the antifibronectin antibody to the subcellular fibronectin coating would be presumed to require "open" spaces or channels large enough to permit passage of immunoglobulin molecules. The monolayers were then stained with the avidin–biotin immunofluorescence technique and examined for fluorescence. Although fluorescence was present after mechanical trauma to the MDCK monolayers, there was no visible fluorescence present after neutrophil migration or H_2O_2 exposure. Thus, over these time periods, neither H_2O_2 exposure nor PMN migration caused sufficient disruption of the MDCK cell monolayer to enable the penetration of fibronectin antibodies.

Discussion

The inflammatory response involves both the accumulation of neutrophils at a site of inflammation and an increase in epithelial and endothelial permeability. Through a series of experiments we have sought to investigate the mechanism of neutrophil accumulation and the relationship between that accumulation and alterations in permeability.

Previous investigators have shown that a decrease in the electrical resistance across an epithelium reflects an increase in permeability. In "leaky" epithelia (those with an electrical resistance of less than 1000 ohms/sq cm) greater than 75% of current flow occurs via paracellular, rather than transcellular pathways,[28] suggesting that a decrease in resistance across a MDCK monolayer probably reflects a change in intercellular junctions. This is further suggested by data from Milks et al demonstrating that permeability for horseradish peroxidase into intercellular junctions correlated with changes in conductance[21] and from Martinez-Palomo et al showing that the decreased electrical resistance across a MDCK monolayer that occurs with Ca^{+2} depletion is clearly the result of the opening in intercellular junctions.[18] Furthermore, Welch et al demonstrated an association between a decrease in the resistance of an epithelial monolayer and an increase in mannitol flux across the monolayer.[38] Accordingly, our experiments are based on the assumption that an alteration in the electrical resistance of a MDCK monolayer represents an increase in epithelial permeability secondary to the opening of intercellular junctions.

Initially we evaluated these phases of neutrophil accumulation: neutrophil activation, neutrophil migration, and neutrophil-epithelial interaction after migration. The first experiments demonstrated that the activation of neutrophils alone did not affect epithelial permeability. This, perhaps, could have been anticipated. Neutrophil-mediated epithelial and endothelial cell injury appears to be dependent on neutrophil adherence and the degree of neutrophil adherence has been shown to be highly variable depending on the epithelium or endothelium studied.[33] Stimulated neutrophil adherence to MDCK monolayers has been shown to be significantly less than that seen with other types of epithelium (M. Tonneson, personal communication). Scanning of the MDCK monolayers in our experiment confirmed this observation. This could also explain the difference between our investigation of the effect of activated neutrophils on rat Type II cell monolayers where we found a decrease in resistance[36] and our work here with MDCK cells. The stimulated adherence of neutrophils to rat Type II cells is markedly greater than to MDCK cells (M. Tonneson, personal communication).

The neutrophil migration experiments confirmed the observation from previous investigators that neutrophil migration alone does not alter epithelial permeability. This suggested that there had to be a mechanism of rapid junctional opening and resealing which was activated either by the neutrophil or by the epithelium in response to the neutrophil.

We have herein demonstrated that a product of

activated neutrophils, H_2O_2, will markedly decrease transepithelial electrical resistance. The initial phase of this H_2O_2-induced decrease in resistance was reversible by the addition of catalase. The return of the resistance to baseline was rapid and appeared to be protein synthesis-dependent. We were surprised that such a rapid mechanism would be dependent on protein synthesis. The rapid Ca^{+2}-dependent junctional resealing described by Martinez-Palomo et al was independent of protein synthesis,[18] and we anticipated that there would not be two independent junctional resealing methods. However, other investigators have suggested that under certain culture conditions the formation of MDCK cell junctions may be protein synthesis-dependent, although the time course of that process was not investigated.[18]

We speculated, then, that neutrophils stimulated by a chemoattractant could produce hydrogen peroxide, which would open intercellular junctions and endogenously produced catalase could implement the junctional resealing following neutrophil migration. However, neutrophil migration was not affected by the presence of catalase. Futhermore, epithelial permeability was not altered when neutrophils migrated in the presence of cycloheximide. This suggests that the intercellular junctional opening and resealing which is presumed to occur during neutrophil migration does not use the protein synthesis-dependent hydrogen peroxide mechanism that we have herein described.

We further demonstrated that neutrophil accumulation beneath an epithelial monolayer following migration through it resulted in an increased permeability of the epithelium. This decrease in resistance was not associated with any visible lifting or disruption of the monolayer and was not prevented by the presence of catalase or AAPVCK. The decrease in resistance occurred despite the migration of relatively few neutrophils through the MDCK monolayers grown on 0.45 μ collagen-coated filters. This is in contrast to data from Milk et al, which shows no change in resistance when neutrophils migrated through a MDCK monolayer grown on uncoated 0.45-μ pore size filters.[22] Multiple features could account for this difference. It has been shown that cells grown on a collagen matrix adhere tightly to that matrix; so they would be less likely to form blebs.[2] Thus, there would be less potential space for migrating neutrophils in our system where monolayers were grown on a collagen matrix. Furthermore, neutrophils have been shown to be more activated (as measured by production of elastase) when migrating through collagen-coated filters as opposed to uncoated filters.[6] In our system, then, relatively few neutrophils could potentially produce large amounts of oxidants and proteases which could account for the alterations in resistance. The inability of catalase or AAPVCK to prevent the resistance in this setting does not eliminate oxidants or proteases as the mediators of injury. There is no way to document whether the catalase or AAPVCK penetrated beneath the monolayer to where the neutrophils were trapped. It is also possible that less chemoattractant could be reaching the apical surface of the monolayers grown on collagen-coated 0.45-μ filters.

We speculate, then, that in acute inflammation the epithelium remains intact during initial neutrophil activation and migration. However, if the neutrophils are subsequently allowed to accumulate beneath the epithelium and are further activated, epithelial injury will occur.

This sequency of events has been suggested by other investigators. Milks and Cramer have clearly shown that neutrophil migration across a cultured epithelial monolayer can occur without altering epithelial permeability.[22] During the process of transepithelial migration, there was no apparent leakage of either horseradish peroxidase or lanthanum suggested that the neutrophil "squeezed" through intercellular junctions, which rapidly sealed behind them. However, if the chemotactic gradient was reversed after the initial neutrophil migration through an epithelium so that the neutrophils reemigrated through the epithelium, the permeability of the epithelium transiently increased.[22] This model of prolonging neutrophil–epithelial contact lends further credence to our hypothesis that the effect of activated neutrophils on epithelial permeability depends, at least in part, on the duration of their interactions.

Similar results have been obtained *in vivo*. In intact rabbits it has been shown that both introduced and intravascularly generated chemotactic factors cause neutrophil sequestration in the lung without a detectable increase in alveolar permeability.[10,37] The addition of a second stimulus (ie, intubation, surgery) caused the migration of neutrophils into the alveoli, but permeability remained unaltered.[37] Only the subsequent addition of a more severe insult such as hypoxia was associated with an increase in alveolar permeability in addition to the neutrophil influx. Furthermore, Johnson and Ward demonstrated in rats that catalase did not alter the migration of neutrophils into the alveoli, but did suppress the increased lung permeability associated with immune complex lung injury. They speculated that in immune complex disease, neutrophils would migrate in response to chemotactic factors and, once at the inflammatory site, would be activated by immune com-

plexes to produce H_2O_2, which could result in cellular injury.[13]

Thus, *in vivo* and *in vitro*, neutrophil migration is not associated with epithelial injury and does not appear to be dependent on oxidants. Our work suggests that neutrophil migration does not occur via a protein synthesis-dependent mechanism of H_2O_2 catalase-facilitated junctional opening and resealing, which we have herein described. We suggest that for a full understanding of the process of acute inflammation, the interaction between neutrophils and epithelia which allows for neutrophil migration needs to be elucidated.

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