

Platelet activating factor compromises airway epithelial defense functions

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Background: The mechanism of disruption of the epithelial defense function observed in asthmatic airways is considered to be largely the result of mediators involved in allergic responses. Platelet activating factor (PAF) might be a key mediator involved in this mechanism.

Objective: This study was designed to determine whether PAF is capable of compromising the epithelial defense functions, such as tight junctional barriers and the mucociliary system.

Methods: A total of 120 healthy rabbits were used. Twenty of them were used as normal controls. Eighty rabbits were treated with inhalation of 10 ml of PAF (200 µg/ml), and 20 animals were used for the examination of epithelial defense functions of the trachea at 1, 10, 20, and 30 days after inhalation of PAF. Epithelial defense functions of the trachea were evaluated by ciliary activity, mucociliary transport velocity, epithelial permeability to fluorescein isothiocyanate dextran (70,000 d), and electron microscopy of the epithelial structure.

Results: PAF inhalation induced a significant decrease in ciliary activity and mucociliary transport velocity, which persisted for up to 20 days. PAF inhalation also caused a significant 7.4-fold increase in epithelial permeability to fluorescein isothiocyanate dextran at 1 and 10 days. This increased epithelial permeability returned to the normal level 20 days after PAF inhalation. However, electron microscopy demonstrated no apparent evidence of epithelial shedding.

Conclusions: PAF-induced prolonged dysfunction of both the epithelial junctional barrier and the mucociliary system may allow enhanced entry of allergen molecules, as well as bronchoactive agonists to the airway parenchyma and may also significantly contribute to an increased airway responsiveness. (*J Allergy Clin Immunol* 1997;100:520-6.)

Key words: Epithelial permeability, ciliary activity, mucociliary clearance, trachea, platelet activating factor

The luminal surface of the airways is lined by a layer of epithelial cells, which forms the interface between the airway parenchyma and the external environment. The principal role of the airway epithelium is as a protective

Abbreviations used

BSA:	Bovine serum albumin
FD-70s:	Fluorescein isothiocyanate-dextran
lyso:	1-alkyl-2-lyso-sn-glycero-3-phosphocholine
PAF:	Platelet activating factor
Papp:	Apparent permeability coefficients

barrier against the invasion of potentially noxious environmental molecules (e.g., environmental allergens). This barrier role is considered to be a major contribution of the airway epithelium to the respiratory defense. Most hydrophilic allergen molecules are believed to pass predominantly through the paracellular route between the epithelial cells and diffuse from the luminal space to the airway parenchyma. At the apical surface, however, the epithelial cells attach to each other by tight junctions, which serve as an effective barrier in restricting the permeation of allergen molecules from the luminal space to the airway parenchyma.¹ The mucociliary transport system also serves as a protective mechanism of the airways, together with the junctional complex barrier. Inhaled allergen molecules entrapped in airway mucus could be propelled toward the mouth by a coordinated beating of the cilia and thus be removed from the airway.

By virtue of the barrier formed by the tight junction and cleaning by the mucociliary clearance, it is likely that the airway epithelium, if integral, is able to segregate environmental allergen molecules within the airway lumen and to play a vital role in a variety of airway diseases. However, epithelial damage, or shedding of airway epithelium, is often regarded as a hallmark of asthma.²⁻⁴ Disruption of epithelial integrity compromises the barrier function of the tight junctions. A more permeable epithelium no longer maintains a barrier function against allergen molecules and may result in enhanced entry of inhaled allergen molecules into the airway tissue. The ciliated cell appears to be the cell type most frequently destroyed in the asthmatic airway. Damaged ciliated cells and disappearance of cilia where they normally exist cause deterioration of mucociliary clearance. Such impaired mucociliary clearance could not efficiently eliminate allergens and low, as well as high, molecular weight molecules from the airway. Therefore damaged epithelium in asthmatic airways may allow easy access of allergens to the submucosal mast cells in allergic asthma.

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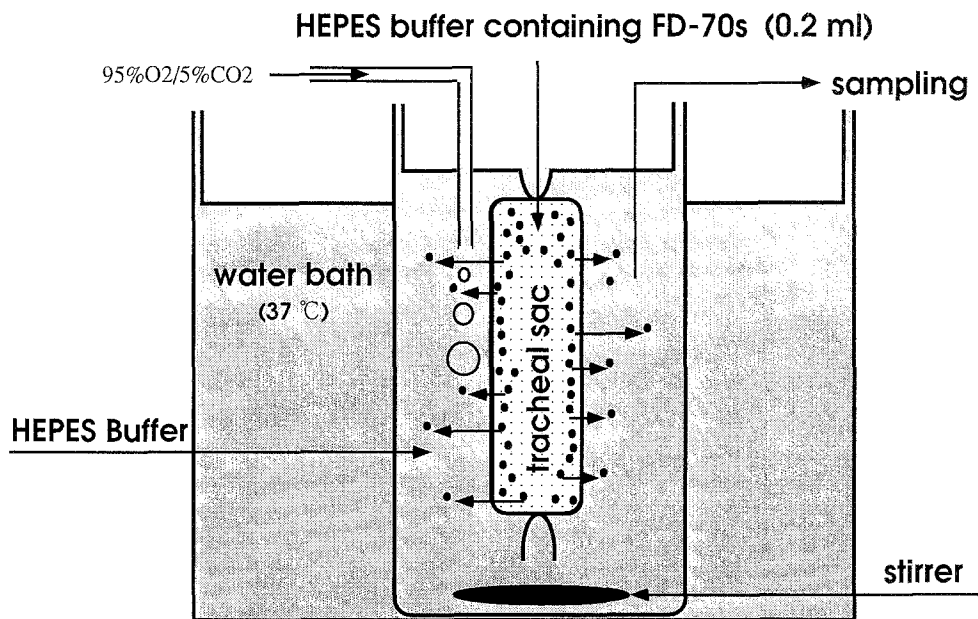


FIG. 1. Schematic presentation of in vitro tracheal sac method. *HEPES*, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid.

The mechanism of disruption of the epithelial defense function observed in asthmatic airways is uncertain but is believed to be largely the result of mediators involved in allergic responses. Histamine and leukotrienes were previously considered to play key roles in mediating allergic responses. Another substance that is receiving increasing attention as a mediator involved in the allergic inflammatory process is platelet activating factor (PAF). PAF induces the aggregation of a wide range of inflammatory cells, including eosinophils, and potentially activates eosinophils. The release of basic proteins such as major basic protein and eosinophil cationic protein from eosinophils can lead to prolonged damage of airway epithelium, such as is observed in the airways of patients with asthma.⁵⁻⁷ In addition, PAF is able to induce a depression of ciliary activity⁸ and an impaired mucociliary clearance,⁹ which is also a feature of asthma.¹⁰ Therefore PAF might be a key mediator involved in disruption of the epithelial defense function observed in asthmatic airways. This study was designed to determine whether PAF is capable of compromising the barrier function of the tight junctions and mucociliary clearance.

METHODS

Groups

One hundred forty healthy white rabbits (average body weight, 2.0 kg) were divided into four groups. Twenty rabbits were used as normal controls (normal group). Twenty rabbits were treated with 10 ml of nebulized phosphate buffer solution containing 5% bovine serum albumin (BSA) delivered by an ultrasonic nebulizer (NE-U-12, Omuron Co. Ltd., Tokyo, Japan) (control group). Another 20 rabbits were also treated with 10 ml of nebulized phosphate buffer solution containing 1-alkyl-

2-lyso-sn-glycero-3-phosphocholine (lyso)-PAF (Funakoshi Co. Ltd., Japan) solution (200 µg/ml), which was dissolved in phosphate buffer solution containing 5% BSA (lyso-PAF group). The animals in the control group and the lyso-PAF group were used for the examination of epithelial defense functions of the trachea 24 hours after the completion of nebulization. The remaining 80 rabbits were treated with 10 ml of nebulized PAF (Funakoshi Co. Ltd., Japan) solution (200 µg/ml), which was dissolved in phosphate buffer solution containing 5% BSA (PAF group). The animals in the PAF group were further divided into four groups of 20 animals each, and each group was used for the examination of epithelial defense functions of the trachea 1 day, 10 days, 20 days, and 30 days after the inhalation of PAF.

Epithelial defense functions were evaluated by the examination of ciliary activity, mucociliary transport velocity, epithelial permeability, and epithelial structure.

Examination of ciliary activity

Six animals from each group were used for examination of ciliary activity in the tracheal mucosa. The animals were killed by decapitation after administration of general anesthesia, and a mucosal specimen (approximately 50 µm in thickness) was obtained from the trachea of each animal. Each mucosal sample was washed in physiologic saline solution to completely remove any secretion present and then placed and sealed in a chamber containing 3 ml of RPMI-1640 medium (Gibco, Grand Island, N.Y.).^{11, 12} The most active ciliated cell of each sample was determined under a phase-contrast microscope (Nikon, Japan), and the ciliary activity (beats per minute) of the ciliated cell from each culture was determined in the RPMI-1640 medium at an ambient temperature of 30°C by using our direct and quantitative photoelectric method.¹³ The methodology and justification of the method used in this study have been described elsewhere.¹³ In brief, changes in rays of light refracted by ciliary motion were projected onto a cadmium

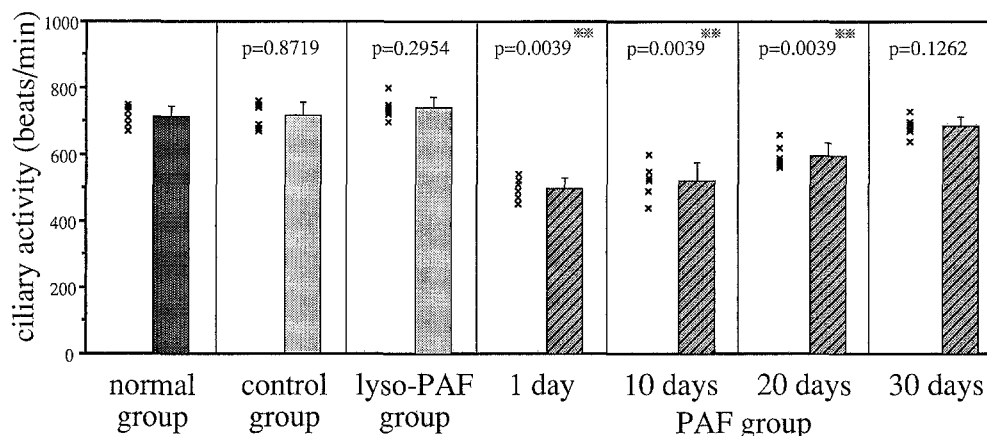


FIG. 2. Ciliary activity in the tracheal mucosa. There are no significant differences in ciliary activity among the normal, control, and lyso-PAF groups. The level of ciliary activity in the PAF group at 1, 10, and 20 days is significantly lower than that in the normal group but returns to the level of the normal group at 30 days. *P* values are significant when compared with the normal group. ****p* < 0.01.

sulfite photoreceptive plate through a pinhole, and signals received by the plate were fed to a pulse-wave pickup (Nihon Koden, Japan) and then to a highly sensitive DC amplifier (Nihon Koden, Japan). Finally, these signals were fed to an oscilloscope (VC-8, Nihon Koden, Japan), and ciliary beats per minute were automatically calculated by an image analysis system.

Examination of mucociliary transport velocity

Six animals from each group were used for the epithelial permeation test. The mucociliary transport velocity of the trachea was determined by an endoscopic method. In brief, each rabbit was deeply anesthetized with pentobarbital sodium and was kept in the supine position. A rigid bronchoscope (inside diameter, 4.0 × 4.9 mm) was inserted into the trachea, and fluorescent microspheres (diameter, 230 nm; Polyscience Inc., Warrington, Pa.) were sprayed onto the tracheal mucosa through the bronchoscope. The movement of the fluorescent vesicles was monitored for 5 minutes by the use of a charge-coupled device camera equipped with a bronchoscope. The mucociliary transport velocity of the trachea (millimeters per minute) was calculated by the analysis of the video imaging.

Epithelial permeation test

Six animals from each group were used for the epithelial permeation test. Permeation tests were carried out by using noninverted sacs (2 cm segment) prepared with excised tracheal tissue from rabbits at 37°C (Fig. 1). N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer solution (0.2 ml, pH 7.4) containing fluorescein isothiocyanate-dextran (FD-70s; molecular weight, 70,000 d) was infused into the sac (mucosal side), and the sac was placed in serosal medium (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer solution [7 ml, pH 7.4]), which was aerated with 95% O₂ and 5% CO₂. Samples (1 ml) were taken from the serosal medium at predetermined times, and the same volume of solution was added to the cell to maintain the original volume. The concentrations of FD-70s were determined by a spectrofluorometer (FR-540, Shimadzu, Japan). Apparent permeability coefficients (Papp) on FD-70s from mucosal side to serosal side were estimated from the

steady-state flux (linear portion) of a number of the cumulative amounts of permeation versus time.

Examination of epithelial structure

Two animals from each group were used for examination of the epithelial structure. At the time of death, mucosal samples from the trachea were obtained. Samples were immediately immersed in 2.5% cold glutaraldehyde in 0.1 mol/L phosphate buffer solution and fixed for 2 hours. They were transferred to a buffer solution for washing and postfixed by 2% osmium tetroxide in the same buffer. After dehydration in ethanol, samples were embedded in epoxy resin. Ultrathin sections were obtained with an ultramicrotome and examined under an H-300 transmission electron microscope (Hitachi).

Statistical analysis

Data from the different groups were statistically analyzed by the Mann-Whitney U test, and the difference was considered significant when the *p* value was less than 0.05.

RESULTS

Ciliary activity

The level (mean ± SD) of ciliary activity in the normal group was 715 ± 31 beats/min (range, 670 to 750 beats/min). The levels of ciliary activity in the control group and the lyso-PAF group were 715 ± 40 beats/min (range, 670 to 760 beats/min) and 740 ± 34 beats/min (range, 700 to 800 beats/min), respectively. Neither the ciliary activity in the control group nor the ciliary activity in the lyso-PAF group was significantly different from that in the normal group (normal group vs control group: *p* = 0.8719; normal group vs lyso-PAF group: *p* = 0.2954). In contrast, the level of ciliary activity in the PAF group at 1 day (497 ± 33 beats/min) was significantly lower than that in the normal group (*p* = 0.0039). Ciliary activity increased thereafter, but the PAF group demonstrated a lower level of ciliary activity at 10 days and 20 days than the normal group (10 days: 522 ± 54

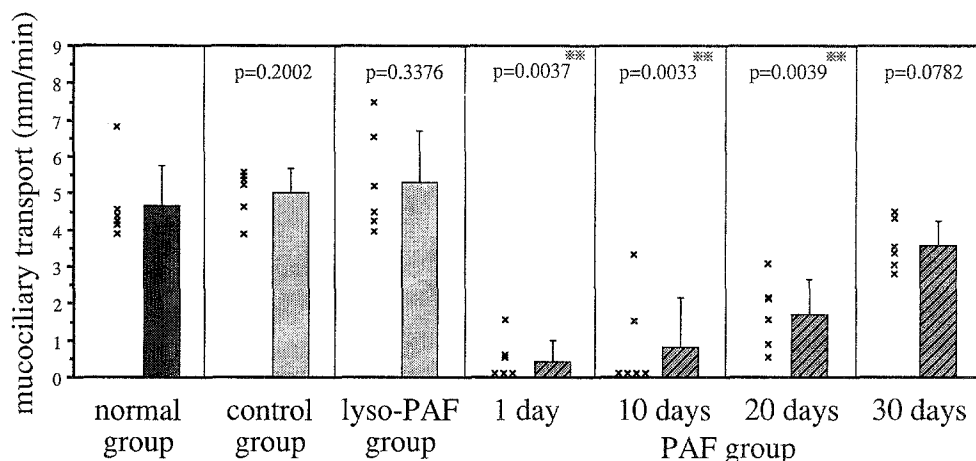


FIG. 3. Mucociliary transport velocity in the tracheal mucosa. There are no significant differences in mucociliary transport velocity among the normal, control, and lyso-PAF groups. The level of mucociliary transport velocity in the PAF group at 1, 10, and 20 days is significantly lower than that in the normal group but returns to the level of the normal group at 30 days. *P* values are significant when compared with the normal group. ****p* < 0.01.

beats/min, *p* = 0.0039; 20 days: 597 ± 37 beats/min, *p* = 0.0039). At 30 days, the level of ciliary activity in the PAF group was 685 ± 30 beats/min, which was not significantly different from that of the normal group (*p* = 0.1262, Fig. 2).

Mucociliary transport velocity

The level (mean ± SD) of mucociliary transport velocity in the normal group was 4.67 ± 1.08 mm/min (range, 3.88 to 6.82 mm/min). The levels of mucociliary transport velocity in the control group and the lyso-PAF group were 5.02 ± 0.65 mm/min (range, 3.89 to 5.57 mm/min) and 5.31 ± 1.41 mm/min (range, 3.96 to 7.50 mm/min), respectively. There were no significant differences between mucociliary transport velocity in the normal group and that in the control group (*p* = 0.2002). In contrast, the level of mucociliary transport velocity in the PAF group at 1 day (0.41 ± 0.57 mm/min) was significantly lower than that in the normal group (*p* = 0.0037). Mucociliary transport velocity increased thereafter, but the PAF group demonstrated a lower level of mucociliary transport velocity at 10 days and 20 days than the normal group (10 days: 0.79 ± 1.36 mm/min, *p* = 0.0033; 20 days: 1.71 ± 0.93 mm/min, *p* = 0.0039, respectively). At 30 days, the level of mucociliary transport velocity in the PAF group was 3.56 ± 0.68 mm/min, which was not significantly different from that of the normal group (*p* = 0.0782, Fig. 3).

Epithelial permeability

The level (mean ± SD) of Papp in the normal group was 4.05 ± 1.08 cm/min × 10⁻¹⁰ (range, 1.39 to 5.57 cm/min × 10⁻¹⁰). The levels of Papp in the control group and the lyso-PAF group were 6.40 ± 2.12 cm/min × 10⁻¹⁰ (range, 3.81 to 8.89 cm/min × 10⁻¹⁰) and 6.02 ± 2.12 cm/min × 10⁻¹⁰ (range, 2.55 to 8.29 cm/min × 10⁻¹⁰), respectively.

Neither the Papp in the control group nor the Papp in the lyso-PAF group was significantly different from that in the normal group (normal group vs control group: *p* = 0.0921; normal group vs lyso-PAF group: *p* = 0.0782). In contrast, the level of Papp in the PAF group at 1 day (19.16 ± 9.41 mm/min) and at 10 days (18.13 ± 11.37 cm/min × 10⁻¹⁰) was significantly higher than that in the normal group (1 day, *p* = 0.0039; 10 days, *p* = 0.0374). Papp decreased thereafter, and the level of Papp in the PAF group at 20 and 30 days was not significantly different from that in the normal group (20 days, 2.15 ± 1.19 cm/min × 10⁻¹⁰, *p* = 0.0782; 30 days, 3.38 ± 2.69 cm/min × 10⁻¹⁰, *p* = 0.6310; Fig. 4).

Epithelial structure

No abnormal findings were observed in the epithelium of the normal and control groups. Sporadic appearance of inflammatory cells including eosinophils was observed in the submucosa of the animals in the PAF group when they were killed at 1 to 20 days after PAF treatment. However, shedding of epithelial cells was hardly seen in the tracheal mucosa of the PAF group. The tight junctions of the PAF group were intact under transmission electron microscopy (Fig. 5).

DISCUSSION

Our study examined the epithelial defense functions of the trachea after inhalation of PAF. The airway epithelium has a variety of defense functions. Of them, the barrier function of tight junctions and the clearance function of the mucociliary system are primitive and nonspecific but are considered to be the most important. Therefore our study was exclusively focused on the functions of the epithelial barrier and the mucociliary system.

The airway epithelial permeability in animals was previously evaluated by the use of various methods.

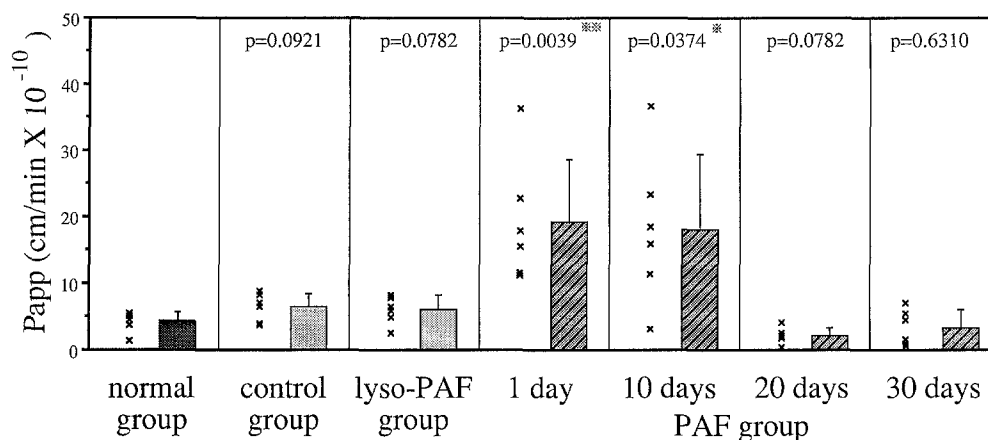


FIG. 4. Epithelial permeability in the tracheal mucosa. There are no significant differences in epithelial permeability among the normal, control, and lyso-PAF groups. The level of epithelial permeability in the PAF group at 1 and 10 days is significantly higher than that in the normal group but returns to the level of the normal group at 20 and 30 days. *P* values are significant when compared with the normal group. **p* < 0.05. ****p* < 0.01.

Boucher et al.¹⁴ and Hulbert et al.¹⁵ developed an epithelial permeability test in animals, in which horseradish peroxidase (molecular weight, approximately 40,000 d) was delivered through a tracheostomy tube, and epithelial permeability was assessed from its movement through the epithelium as revealed by electron microscopy and by its appearance in the blood. Ranga et al.,¹⁶ making use of a similar technique, examined airway epithelial permeability of carbon 14-labeled mannitol (approximately 182 d), tritiated dextran (approximately 10,000 d), and horseradish peroxidase (approximately 40,000 d) in sensitized guinea pigs. However, quantitative detection of tracers in plasma may not always represent the epithelial permeability because barriers of vascular endothelial cells are involved in such a testing method. Indeed, some studies compared the *in vivo* epithelial permeability with the *in vitro* epithelial permeability from the same individuals and showed that the *in vitro* permeability was very homogeneous, whereas the *in vivo* permeability was highly variable among individuals studied.¹⁷

In vitro tests for examination of airway epithelial permeability have used either strips or tubular segments of airways. The two preparations differ greatly in their capacity to demonstrate the properties of the epithelial barrier. The epithelial barrier of the airway strips is substantially attenuated because all surfaces, including cut surfaces, are simultaneously exposed to tracers. By contrast, the luminal surface of the tubular preparations is lined by an intact epithelium and remains a compartmentalized barrier.¹⁸

In this study, to quantitatively examine the barrier function of the epithelium, we used an *in vitro* tracheal sac method, a permeation test of a type of tubular preparations. This method can quantitatively determine the epithelial permeability through the tight junctions, the methodology and justification of which have been

described elsewhere.¹⁹ Because the primary purpose of this study was to determine the epithelial barrier function against allergen molecules involved in respiratory allergic disorders, we chose FD-70s as tracers the molecular weight of which is approximately 70,000 daltons. Because these tracers are hydrophilic and cannot penetrate the epithelium through the cellular route, our permeation test represents the permeability of macromolecules through pores in tight junctions exclusively.

This study has clearly demonstrated that inhalation of PAF solution produced a significant 4.7-fold increase in epithelial permeability to FD-70s at 1 day. PAF was dissolved in phosphate buffer solution containing 5% BSA. Neither phosphate buffer solution containing 5% BSA nor inactive metabolite lyso-PAF induced a significant increase in epithelial permeability. Therefore PAF itself appears to be responsible for the increased permeability observed 1 day after inhalation of PAF solution. Because no epithelial injury was observed in the PAF group, an increase in epithelial permeability to FD-70s was not due mainly to penetration through epithelial defects but mainly to an increased penetration through tight junctions. An inverse relationship has been established between epithelial permeability and molecular weight.²⁰ Because the molecular weights of major allergens involved in asthma and allergic rhinitis, such as house dust mites and pollens, are smaller than the molecular weight of FD-70s, PAF could enhance entry of these allergens into the airway submucosa where mast cells exist. If such a phenomenon occurs in individuals with allergic asthma, PAF-induced disruption of epithelial barriers could result in easy access of allergen molecules to the relevant IgE antibodies on mast cells. This contact of allergens and related IgE antibodies on mucosal mast cells leads to a further release of PAF in the airway mucosa, which may compromise further dysfunction of epithelial barriers. The justification of our

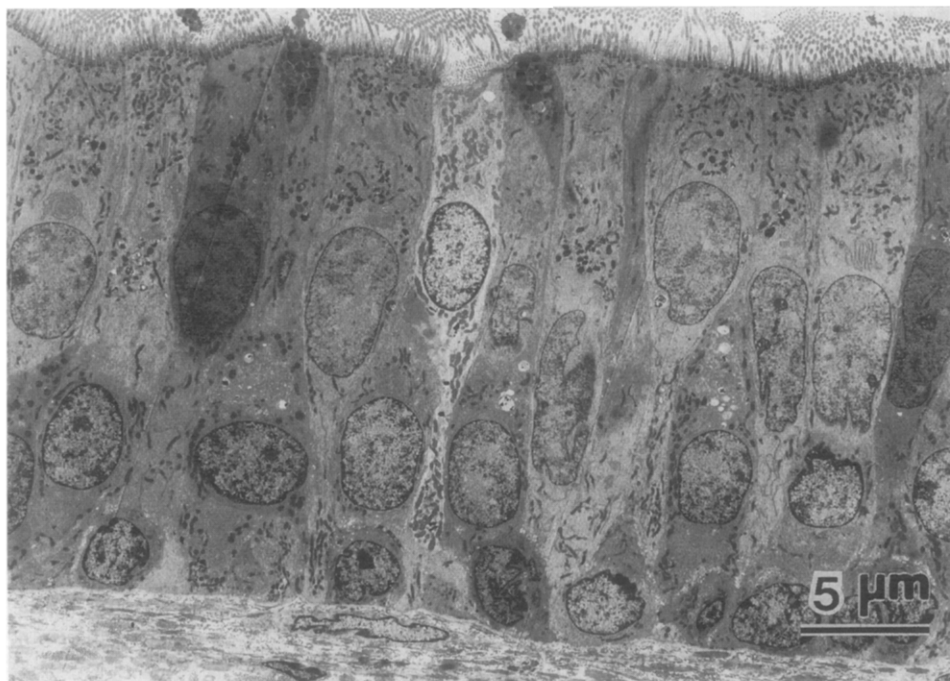


FIG. 5. Electron photomicrograph showing epithelial structure in the PAF group at 1 day. There are no pathologic findings in the epithelium.

hypothesis may be supported by recent freeze-fracture findings showing that there is abnormal tight junction integrity in the asthmatic airway epithelium.²¹

Airway hyperresponsiveness is a characteristic abnormality in asthma, which is a state of increased sensitivity to many different stimuli, such as bronchoactive agonists and chemical irritants. The mechanisms of airway hyperresponsiveness in asthma remain to be conclusively established. Several investigations have proposed that epithelial shedding may intrinsically contribute to airway hyperresponsiveness.⁴⁻⁶ Epithelial shedding is often regarded as a hallmark of asthmatic airways, and a close association was indeed reported between epithelial shedding and hyperresponsiveness.⁴⁻⁶ However, several authors have reported the absence of such loss of epithelial cells in asthmatic airways.^{22, 23} Therefore hyperresponsiveness in asthma might be the result of mechanisms other than epithelial sloughing.

In this study, inhalation of PAF increased the tight junction permeability to macromolecules of 70,000 d without apparent morphologic evidence of epithelial damage. Because most bronchoactive agents are much smaller than FD-70s in molecular weight, they more easily pass the pores in tight junctions and reach the smooth muscle. An increased permeability of tight junctions by PAF may be a possible mechanism of hyperresponsiveness. Indeed, inhaled PAF, unlike other mediators of asthma, is able to cause increased bronchial responsiveness in animals and normal subjects, as well as in subjects with asthma.²⁴⁻²⁶

We also studied the effect of PAF inhalation on the function of the mucociliary system. Because the muco-

ciliary function is composed of the ciliary activity and the mucus blanket covering it, mucociliary dysfunction could result from reduced ciliary activity, abnormalities of the physiologic properties of airway secretions, or both. We therefore examined both ciliary activity and mucociliary transport velocity in the rabbit trachea after inhalation of PAF. In our previous study, PAF alone never affected ciliary activity in the tracheal epithelium, but PAF reduced ciliary activity in the presence of alveolar macrophages.⁸ This study has clearly demonstrated that inhalation of PAF caused significant depression of both ciliary activity and mucociliary transport velocity in the trachea. Such mucociliary dysfunction could not efficiently eliminate inhaled allergens from the airway.

Therefore dysfunction of both the epithelial junctional barrier and the mucociliary system may allow enhanced entry of allergen molecules and bronchoactive agonists to the airway parenchyma and may also significantly contribute to increased airway responsiveness. We also studied epithelial defense functions for up to 30 days after PAF inhalation. Junctional dysfunction and mucociliary dysfunction persisted for up to 10 and 20 days after inhalation of PAF, respectively. Single inhalation of PAF does not result in permanent dysfunction of epithelial defense, but its influence persists for a considerably long period. In addition, the bronchial epithelium of patients with asthma may be more susceptible to PAF than that of normal subjects,²⁷ and the influence of PAF may cause a more sustained impairment of epithelial defense functions.

In conclusion, this study has demonstrated a substantial influence of PAF on the airway epithelial defense

functions. This study supports the concept that PAF plays an important role in both the epithelial permeability of allergen molecules and airway hyperresponsiveness.

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