Neutrophils enhance clearance of necrotic epithelial cells in ozone-induced lung injury in rhesus monkeys

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Neutrophils enhance clearance of necrotic epithelial cells in ozone-induced lung injury in rhesus monkeys. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L1190–L1198, 1999.—To test the hypothesis that neutrophil influx is important for the removal of necrotic airway epithelial cells, rhesus monkeys were treated with a function-blocking monoclonal antibody (MAb) against CD18 followed by exposure to ozone or filtered air. CD18 MAb-treated, ozone-exposed monkeys showed a significant inhibition of neutrophil emigration and an accumulation of necrotic airway epithelial cells. In a subsequent experiment, monkeys were given CD18 MAb or an isotype control immunoglobulin before ozone or filtered-air exposure. Complement 5a was instilled into lobes of the right lung at the end of the exposure. Lavage neutrophils were significantly elevated in the right lobes compared with those in the contralateral left lobes; consequently, there were significantly fewer necrotic cells in the airways of the right lung, whereas large aggregations of necrotic cells were observed in the contralateral airways of the left lung. These data indicate that neutrophil influx in ozone-induced injury in primates is CD18 dependent and that neutrophils contribute to the repair of airway epithelium by removal of injured epithelial cells.

SHORT-TERM INHALATION of ozone in rhesus monkeys causes damage to pulmonary epithelial cells in the anterior nasal cavity, trachea, and respiratory bronchioles. Acute injury results in necrosis of ciliated cells, deciliation and degranulation of secretory cells in conducting airways, and necrosis of type I cells and ciliated cells in respiratory bronchioles. Epithelial necrosis in the respiratory bronchioles of monkeys occurs as early as 4 h and is maximal between 12 and 24 h after the initiation of ozone exposure, and there is a strong relationship between epithelial necrosis and the emigration and retention of neutrophils at all levels of the tracheobronchial tree (2, 12, 19). The response of the monkey respiratory bronchiolar epithelium to a necrotizing dose of ozone (0.96 part/million (ppm)) for 8 h has been carefully documented morphometrically (12). Furthermore, the respiratory bronchiole is the region of the monkey airway that shows persistent changes with long-term ozone inhalation (9).

The role of the adhesion molecule intercellular adhesion molecule (ICAM)-1 in antigen-induced airway inflammation has been studied in nonhuman primates. Anti-ICAM-1 antibody (Ab) was highly effective in reducing airway eosinophilia in monkeys after repeated Ascaris challenge (24). Adhesion molecules also appear to be necessary for ozone-induced neutrophil migration into the airway lumen in dogs. Treatment with the anti-Mo1 (CD11b/CD18) Ab significantly limited lavage fluid neutrophils and eosinophils in response to ozone inhalation (3 ppm for 30 min) (14). Mice exposed to a necrotizing dose of ozone (2 ppm) for 3 h showed immunohistochemical staining of ICAM-1 in the trachea and, to a lesser extent, in the lobar and segmental bronchi as early as 3 h after exposure to ozone (21). The expression of ICAM-1 was temporally associated with the influx of myeloperoxidase-positive inflammatory cells, presumably neutrophils. However, in vitro studies suggest that ICAM-1 expression on epithelial cells is not critical to neutrophil adherence after ozone exposure. Monoclonal Abs (MAbs) to CD18, but not to ICAM-1, inhibited adherence of stimulated neutrophils to ozone-exposed primary cultures of rhesus monkey bronchial epithelial cells, primary cultures of human tracheal epithelial cells, or transformed human bronchial epithelial cells (17, 22). Similarly, neutrophil chemotaxis through airway epithelial cell monolayers is CD11b/CD18 dependent (15).

The role of neutrophil CD11b/CD18 function in neutrophil migration into the lung appears to be complex: both CD18-dependent and CD18-independent processes have been implicated (6, 18, 20). Anti-CD18 MAb (60.3) in rabbits markedly reduced neutrophil influx in response to Escherichia coli endotoxin or phorbol 12-myristate 13-acetate, but did not inhibit neutrophil influx in response to Streptococcus pneumoniae or hydrochloric acid (6). One of the stimuli that is CD18 independent for neutrophil recruitment into lung parenchyma is C5a (5, 10). The response to C5a instillation in the adult rabbit is characterized by an accumulation of neutrophils in the alveolar air spaces that emigrate primarily through the capillaries of the interalveolar septa within 4 h after instillation (7).

A dual role for the neutrophil in the epithelial response to ozone-induced acute injury has been identified in vitro. Isolated rat alveolar epithelial cells are capable of forming tight, polarized, sodium-absorbing...
monolayers when cultured on porous Transwell inserts in the presence of serum. Dose-response relationships
and the interactive effects of ozone injury on alveolar epithelial barrier function in vitro have been investigat-
gated with this model system (3). Both the direction and magnitude of the effects of neutrophils on oxidant-
ated impairment of alveolar epithelial barrier function were dependent on ozone dose (4). Monolayers
exposed to either filtered air or 0.1 ppm ozone and subsequently administered neutrophils on their api-
cal surfaces had significantly fewer injured cells at 5 h postexposure and greater transepithelial resistance
values at 24 h postexposure relative to monolayers not subjected to neutrophils. However, this trend with
neutrophils was reversed with increasing ozone concentration (0.2 or 0.5 ppm): significantly more injured cells
at 5 h postexposure and lower transepithelial resistance values at 48 h postexposure relative to monolay-
ers exposed to the same concentration of ozone only.

In this study, we extended our previous investigations in monkeys (12, 19) by testing the hypothesis that
inhibition of neutrophil influx after ozone-induced injury would delay removal of necrotic epithelial cells and
subsequent epithelial repair. We also tested the hypoth-
thesis that C5a-induced recruitment of neutrophils into the lobes on one side (right) of the lung would enhance
removal of necrotic epithelial cells and subsequent epithelial repair on that side compared with that in the
lobes of the contralateral side (left) in CD18 MAb-treated monkeys.

METHODS

Animals

Young male rhesus monkeys weighing 5.1–7.6 kg and 3 yr
8 mo to 3 yr 10 mo in age were randomly assigned to exposure
and/or treatment groups in two experiments as follows: filtered air, filtered air plus CD18 MAb, filtered air plus
isotype control immunoglobulin, ozone, ozone plus CD18
MAB, and ozone plus isotype control immunoglobulin (Table 1). All monkeys were given a comprehensive physical exami-
nation including a chest radiograph and complete blood count
before exposure. The monkeys were transferred to exposure
chambers for a 1-wk acclimatization, during which time they breathed filtered air.

Exposure Regimen

The monkeys were exposed individually in exposure
chambers of 4.2 m3 capacity that were ventilated at a rate of 30
changes/h with chemical, biological, and radiological filtered
air at 24 ± 2°C and 40–50% relative humidity. Oxygen was
passed through a Sanders model 25 ozonizer (Eltez, Ger-
many) to produce ozone. Ozone concentrations were mea-
sured with an ultraviolet ozone monitor (model 1003-AH,
Dasibi Environmental, Glendale, CA) and reported with
respect to the ultraviolet photometric standard. Monkeys
were exposed to 0.0 and 0.8 ppm ozone for 8 h, followed by
postexposure in filtered air for 4, 24, or 48 h (Table 1).

Table 1. Distribution of monkeys after exposure in FA

<table>
<thead>
<tr>
<th>Time After Exposure</th>
<th>Experiment 1 Treatment Group</th>
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<tbody>
<tr>
<td></td>
<td>FA</td>
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<tr>
<td>24 h</td>
<td>3</td>
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<tr>
<td>48 h</td>
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<th>Time After Exposure</th>
<th>Experiment 2 Treatment Group</th>
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<td>FA + CD18 MAb</td>
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<td>4 h</td>
<td>2</td>
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</table>

FA, filtered air; MAB, monoclonal antibody; O3, ozone (0.8 ppm). In experiment 1, CD-18 MAB-treated monkeys were given antibody (2 mg/kg body wt) 16 h before exposure and every 24 h thereafter. In experiment 2, CD-18 MAB- or isotype control immunoglobulin-treated monkeys were given antibody (1 mg/kg body wt) 8 h before exposure. All monkeys had C5a instillation at end of 8 h of exposure to FA or O3 in right middle and caudal lung lobes. All monkeys received an intraperitoneal injection of 5-bromo-2'-deoxyuridine 3 h after exposure (1 h before necropsy).
0.5-µm serial sections for use with a 5-µm physical “disector” to estimate the number of cells per cubic millimeter of respiratory bronchiolar epithelium \(N_v(c/ep)\) with the formula (1) \(N_v(c/ep) = \frac{Q_c}{A_{ep} \cdot H_{dis}}\), where \(Q_c\) is the number of epithelial cell nuclei counted in the reference section that is not intersected by the lookup section, \(A_{ep}\) is the area of the epithelium, and \(H_{dis}\) is the height of the disector in the sample (the sections are 4 µm apart and the two sections are 0.5 µm thick, for a total sample distance of 5 µm). We estimated the surface area of the basement membrane (bm) to the epithelial volume \(V_{ep/bm}\) by point and intersection counting as follows (1): \(V_{ep/bm} = 2L_{bm} \cdot S_{ep}\), where \(L_{bm}\) is intersection of the test line of an eyepiece grid with the epithelial basement membrane and \(S_{ep}\) is the test line length over the epithelium. We normalized the number of epithelial cells by the epithelial basement membrane surface area to allow us to compare epithelial cells between respiratory bronchioles as shown by 

\[ N_v(c/bm) = \frac{N_v(c/ep)}{S_{ep/bm}} \]

where the epithelial volumes are the same and divide to 1. To estimate the mean volume of the nonciliated bronchiolar cell \(V_{ncb}\) in cubic micrometers, we used the formula (1) \(V_{ncb} = \frac{V_{ep/bm}}{N_v(c/ep)}\), where \(V_{ncb}\) is the volume density of the nonciliated bronchiolar cell in the epithelium and \(N_v(c/ep)\) is the number of nonciliated bronchiolar cells per volume of respiratory bronchiolar epithelium. To estimate the arithmetic mean thickness of respiratory bronchiolar epithelium \(t_{ep}\), we used the formula (25) \(t_{ep} = V_{ep} / S_{ep}\), where \(V_{ep}\) is the volume of the epithelium estimated by point counting and \(S_{ep}\) is the surface area of the epithelial basal lamina estimated by point and intersection counting.

**Experiment 2**

Ab treatment. The monkeys were given the CD18 MAb or an isotype control in the cephalic vein at 1 mg/kg body wt 8 h before exposure (Table 1).

C5a bronchoscopy. Immediately at the end of the 8-h exposure period, the monkeys were anesthetized with ketamine (10 mg/kg) administered subcutaneously. For these monkeys, 1 and 1.5 ml of human recombinant C5a (4 µg/ml) in PBS were instilled into the right middle and caudal lobes, respectively, with an Olympus BF-7 pediatric bronchoscope (Olympus America, Melville, NY) (Table 1).

5-Bromo-2′-deoxyuridine injection, necropsy, bronchial wash, toilet samples. After 3 h postexposure in filtered air, the monkeys were anesthetized with ketamine (30–35 mg/kg) administered subcutaneously, injected intra-peritoneally with 5-bromo-2′-deoxyuridine (BrdU; 30 mg/kg; Sigma) dissolved in PBS, and killed 1 h later with a pentobarbital sodium overdose in accordance with the Animal Care Guidelines of the California Regional Primate Research Center (Table 1). BrdU, a thymidine analog incorporated into DNA by cells undergoing DNA replication, is indicative of cell proliferation. The right middle and left cranial lung lobes were instilled with 30 and 60 ml, respectively, of 12 µM ethidium homodimer-1 (Molecular Probes, Eugene, OR) in Waymouth medium for 15 min, lavaged with PBS, and instillation fixed for 30 min in 1% glutaraldehyde-1% paraformaldehyde (cachodlate buffer, 440 mM, pH 7.4). The right and left caudal lobes were lavaged with PBS and fixed by instillation of buffered Formalin fixative (Z-Fix, Anatech) for morphological examination while the trachea and remaining lobes were frozen for immunocytochemistry. The recovery of lavage fluid was 85 ± 7% of the volume infused for all of the caudal lung lobes. The total nucleated cell count was estimated with a Coulter counter (Coulter), and a differential count on a minimum of 300 cells was completed with a cyto spun and Diff-Quik stain. Values are expressed as total neutrophils recovered from lavage of the lobe.

Airway microdissection and confocal and light microscopy. The right middle and left cranial lobes were microdissected beginning at the lobar bronchus (19). The intrapulmonary airways and accompanying parenchyma were split down the long axis of the largest daughter branch or down the axial pathway of the primary airway. An attempt was made to expose as many minor daughter side branches as possible. Each airway was numbered with a binary system that provides a branching history for each segment of the conducting airways. Samples were taken from the costal and mediastinal sides of the proximal and distal airways. Samples included bronchi (8th- and 6th-generation airways for left cranial and right middle lobes, respectively) and first-generation respiratory bronchioles (16th- and 12th-generation airways for left cranial and right middle lobes, respectively). The samples were mounted on coverslips with glue and stained with a second DNA-binding fluorochrome YO-PRO-1 (Molecular Probes). The samples were then immersed in a small petri dish for observation on a Bio-Rad 600 confocal microscope with water-immersion lenses. The eighth subsegmental bronchus and at least two first-generation respiratory bronchioles were digitally captured for each lobe on both the costal and mediastinal sides of the airways. This method of ethidium uptake marks necrotic epithelial cells red (ethidium positive) in a green epithelial background (23).

Semi-quantitative grading system of confocal images. We evaluated the number and extent of ethidium-positive cells in airways in a semi-quantitative fashion with confocal images of microdissected airways without knowledge of exposure group. Scores were based on a one to seven grading scheme where 1 = no ethidium-positive cells, 2 = 1–10 ethidium-positive cells, 3 = 11–20 ethidium-positive cells, 4 = ethidium-positive cells in up to one-fourth of the airway, 5 = ethidium-positive cells in one-fourth to one-half of the airway, 6 = ethidium-positive cells in one-half to three-fourths of the airway, and 7 = ethidium-positive cells in the entire airway.

Histopathology of whole mount airways. To assess the nature of the cells labeled with ethidium homodimer-1, the airways were evaluated by light microscopy. Those regions were removed from whole mounts of the lungs by slicing perpendicular to the long axis of the axial airway after laser scanning was completed. The slices were dehydrated in ethanol and embedded in glycolmethacrylate (Immuno-Bed, Polysciences, Warrington, PA). The blocks were sectioned at 1 µm on a Sorvall JB-4 microtome. After unstained sections were evaluated by confocal microscopy, a serial section was stained with toluidine blue and evaluated by transmission light microscopy with an Olympus Provis microscope.

BrdU cell labeling. Paraffin sections were deparaffinized in xylene, hydrated in a graded series of ethanol, and treated with 0.07 N NaOH to denature the DNA. BrdU was detected by treating the sections with a primary MAb directed against single-stranded DNA containing BrdU (ICN Immunobiologicals, Costa Mesa, CA) with a biotinylated secondary antibody, an avidin-bound peroxidase complex (ABC Vectastain peroxidase mouse IgG kit, Vector Laboratories), and 3,3′-diaminobenzidine (Sigma). The standard controls for the above-described method included 1) substitution of the primary antibody with PBS, 2) substitution of the primary antibody with normal serum, and 3) serial dilutions of the primary antibody incubated with serial tissue sections (culture medium dilutions ran from 1:2 to 1:10,000 in steps). The duodenum served as a positive-labeling control for each monkey.
Statistics. For comparisons between contralateral lobes in experiment 2, we used the paired t-test for bronchoalveolar lavage fluid data and the nonparametric Wilcoxon signed rank test for the semiquantitative grading system. For comparisons of ozone-exposed groups for differences between lobes within groups, we used the separate t-test for bronchoalveolar lavage fluid data and the nonparametric Kruskal-Wallis test for the semiquantitative grading system. We analyzed all other data by ANOVA and Fisher's least significant difference test (SYSTAT 5 for the Macintosh, version 5.2, SYSTAT, Evanston, IL). All data are means ± SE. Significance is accepted at P < 0.05.

RESULTS

Experiment 1

Bronchoalveolar lavage. Bronchoalveolar lavage fluid showed a significant decrease of neutrophils at 24 h and a marked decrease in eosinophils at 48 h in CD18 Ab-treated monkeys exposed to ozone compared with the control ozone-exposed monkeys (Fig. 1). Filtered-air exposed monkeys showed no neutrophils or eosinophils, few lymphocytes, and primarily alveolar macrophages in lavage fluid.

Morphometry of respiratory bronchioles. The significant decrease in lavage fluid neutrophils was even more dramatically reflected by the morphometric measurements of the epithelial compartment (between epithelial cells or attached to their apical surface), whereas no neutrophils were observed at 24 h in CD18 MAb-treated monkeys exposed to ozone compared with a significant increase in control ozone-exposed monkeys (Fig. 2). There was an increase in alveolar macrophages within the epithelial compartment at 24 h in the CD18 Ab-treated monkeys exposed to ozone compared with that in control ozone-exposed monkeys (Fig. 2). The number of necrotic epithelial cells per square millimeter of epithelial lamina was significantly increased at both 24 and 48 h after ozone exposure in CD18 MAb-treated monkeys exposed to ozone compared with that in control ozone-exposed monkeys (Fig. 3). Furthermore, at 24 h in CD18 MAb-treated monkeys, we observed a significant increase in the exposed basal lamina that was devoid of epithelium compared with that in all other groups and times (Fig. 3). In contrast, the arithmetic mean thickness of the respiratory bronchiolar epithelium was significantly increased in the control ozone-exposed monkeys at 24 h compared with that in filtered-air exposed monkeys (Fig. 4). An increased epithelial thickness occurs by increasing the number of cells or their individual cell volumes. Hence, it is noteworthy that there was a significant increase in the number of nonciliated bronchiolar cells in the control ozone-exposed monkeys at 24 h compared with

Fig. 1. Number of cells recovered by bronchoalveolar lavage in monkeys exposed for 8 h to ozone (O3; 0.8 ppm) alone or O3 plus CD18 antibody (Ab) followed by 24 or 48 h in FA. Values are means ± SE. *Significantly greater than all other groups, P < 0.05.

Fig. 2. Number of PMN and MAC in epithelial compartment (between epithelial cells or attached to epithelial cells on luminal surface)/mm2 (N) of epithelial basal lamina in monkeys exposed to FA, O3 (0.8 ppm) alone, or O3 + Ab followed by 24 or 48 h in FA. Values are means ± SE. N, none observed. Significantly greater (P < 0.05) than: *all other groups; ‡FA-exposed monkeys.

Fig. 3. Number of necrotic epithelial cells/mm2 of epithelial basal lamina and surface of exposed basal lamina that is devoid of epithelium (SV) in monkeys exposed for 8 h to O3 (0.8 ppm) or O3 + Ab followed by 24 or 48 h in FA. Values are means ± SE. Significantly greater (P < 0.05) than: *O3 group at 24 h; ‡O3 group at 48 h.
that in all other groups (Fig. 5). In contrast, we observed a significant increase in the volume of nonciliated bronchiolar cells in the control ozone-exposed monkeys at 48 h compared with that in other groups (Fig. 6).

Experiment 2

Bronchoalveolar lavage. Because ozone-induced lung injury in rhesus monkeys appeared to be CD18 dependent for neutrophil recruitment, we used C5a instillation, a stimulus that appears to be CD18 independent, to recruit neutrophils into lobes of the right lung of isotype control- and CD18 MAb-treated monkeys. Bronchoalveolar lavage fluid showed that C5a treatment resulted in recruitment of neutrophils into the right middle lobe of all treatment groups (Fig. 7). Although CD18 MAb inhibited more than half of the C5a-induced neutrophil emigration in filtered air- and ozone-exposed monkeys, there were significantly more neutrophils recovered in lavage fluid from the lobe treated with C5a than from the lobe without C5a (Fig. 7). It is noteworthy that CD18 MAb treatment inhibited most of the neutrophil emigration into the ozone-injured left cranial lung lobe without C5a instillation (Fig. 7). Furthermore, the difference between lobes in the CD18 MAb-treated monkeys was significantly less than between the lobes in the isotype control monkeys (Fig. 7).

BrdU cell labeling. Our goal was to investigate whether epithelial cell migration could cover bare basal lamina after sloughing or removal of injured epithelial cells in the absence of epithelial proliferation. BrdU cell labeling showed no labeled epithelial cells in the respiratory bronchioles or distal bronchi in any of the...
monkeys. The only BrdU cell labeling observed was in bronchus-associated lymphoid tissue in ozone-exposed monkeys, whereas filtered air-exposed monkeys showed no labeling.

Laser confocal microscopy. Laser confocal microscopy showed marked necrotic epithelial cells and layers of cellular debris with the vital dye ethidium homodimer-1 in the left cranial lung lobes of most respiratory bronchioles of CD18 MAb-treated monkeys and in some respiratory bronchioles of the isotype control-treated, ozone-exposed monkeys (Fig. 8). Semiquantitative grading of ethidium-positive cells in the airways confirmed that C5a-treated airways of the right middle lung lobe had fewer injured cells than the comparison left cranial lung lobe that received no C5a (Fig. 9). This proved to be significant for the CD18 MAb-treated monkeys exposed to ozone where the differences were greatest between the two lobes (with and without C5a). Furthermore, the score differences between the lobes of the ozone-exposed CD18 MAb-treated monkeys were significantly greater than those in the ozone-exposed isotype control-treated monkeys (Fig. 9). Sections of airways viewed by laser confocal microscopy confirmed that the left cranial lung lobes of CD18 MAb-treated monkeys exposed to ozone had abundant necrotic epithelial cells and layers of cellular debris along with areas devoid of epithelium, whereas the isotype control-treated monkeys exposed to ozone had squamous mixed with cuboidal epithelium along with some cellular debris in the lumen (Fig. 8, B–D). In contrast, treatment with C5a resulted in an intact epithelium that was primarily cuboidal in nature in all immunoglobulin treatment...
DISCUSSION

The results of this study supported our overall hypothesis that neutrophils contribute to the repair process by removal of ozone-injured airway epithelial cells. Short-term ozone inhalation results in necrosis of ciliated cells, deciliation and degranulation of secretory cells in conducting airways, and necrosis of type I cells and ciliated cells in respiratory bronchioles of monkeys. There is a strong relationship between the degree of necrosis and the emigration and retention of neutrophils at all levels of the tracheobronchial tree (2, 12 19). The lack of neutrophils and eosinophils in lavage fluid and respiratory bronchiole epithelium showed that inhibition of neutrophil and eosinophil recruitment in response to ozone-induced epithelial injury was accomplished with a CD18 MAb (Figs. 1 and 2). These data in monkeys with CD18 MAb after ozone inhalation for 8 h demonstrate that the influx of neutrophils and eosinophils into the airways in response to ozone-injured epithelial cells is primarily CD18 dependent.

These observations in the airways are in contrast to a variety of inflammatory stimuli that are CD18 independent and also include the lung parenchyma (6, 18). One of the stimuli that is CD18 independent for neutrophil recruitment into the lung parenchyma is C5a (5, 10). The response to C5a instillation in the adult rabbit is characterized by an accumulation of neutrophils in the alveolar air spaces that emigrate primarily through the capillaries of the interalveolar septa but not through the vessels in airway walls within 4 h after instillation (7). Furthermore, intravascular infusion of complement fragments induces the release of neutrophils from bone marrow within 7 min of infusion. These neutrophils sequester in the lungs immediately on reaching the pulmonary capillaries and thereby provide an additional source of neutrophils for recruitment into the parenchymal air spaces (13). Monkeys treated with CD18 MAb, exposed to ozone, and given C5a had significantly fewer neutrophils in lavage fluid than monkeys treated with the isotype control, exposed to ozone, and given C5a (Fig. 7). This implies the possibility that in C5a-induced pulmonary inflammation there is a level of neutrophil emigration that will occur whether CD18 is blocked or not; neutrophil accumulation beyond this level is CD18 dependent. This concept is supported by a greater than 50% decrease in lavage fluid neutrophils in filtered air-exposed monkeys treated

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Fig. 9. Semiquantitative grading results of ethidium-positive cells of costal (A) or mediastinal (B) half of respiratory bronchioles (RB) and costal half of proximal bronchi (C) from right middle lobe (+C5a), left cranial lobe (−C5a), and difference between lobes (Δ) in monkeys treated with isotype control or CD18 Ab and exposed for 8 h to O3 or FA followed by 4 h in FA. Values are means ± SE. *Significantly fewer ethidium-positive cells than in contralateral lobe that did not receive C5a, P < 0.03 by Wilcoxon signed rank test. ‡Significantly more cells between lobes in O3+Ab than in O3+isotype control-treated monkeys, P < 0.01 by Kruskal-Wallis test.
with CD18 MAb and C5a compared with filtered air-exposed monkeys treated with the isotype control immunoglobulin and C5a (Fig. 7). However, even with the 50% decrease, lavage fluid neutrophils in filtered air-exposed monkeys treated with the isotype control immunoglobulin and C5a were still greater than those in ozone-exposed monkeys treated with the isotype control immunoglobulin without C5a.

The response of monkey respiratory bronchiolar epithelium to ozone (0.96 ppm for 8 h) has been documented morphometrically (12). No necrotic epithelial cells were observed beyond 24 h after exposure in that study or in this study without Ab (Fig. 3). In contrast, CD18 MAb-treated monkeys had significantly greater numbers of necrotic cells 48 h after ozone exposure. This difference in the respiratory bronchiolar epithelium at 24 h was also accompanied by a significant increase in the number of nonciliated bronchiolar epithelial cells per square millimeter of epithelial basal lamina and the arithmetic mean thickness of the epithelium in ozone-exposed monkeys without Ab (Figs. 4 and 5). These observations along with no change in the volume of nonciliated bronchiolar epithelial cells (Fig. 6) show that the increased thickness of the epithelium is accomplished entirely by increases in the number of nonciliated bronchiolar epithelial cells. Bronchi show the greatest epithelial cell labeling with tritiated thymidine, and respiratory bronchioles show very little epithelial cell labeling in monkeys after ozone exposure (2, 12); we conclude that restitution of respiratory bronchiolar epithelium 24 h after injury occurs primarily by migration of proliferating epithelial cells from the bronchi. It is also apparent that CD18 MAb-treated monkeys exposed to ozone lack these beneficial epithelial changes in respiratory bronchioles because of the retention of degenerating and necrotic epithelial cells. Hence it appears that removal of injured epithelial cells is an important step in repair of the epithelium.

It is noteworthy that 24 h after exposure in monkeys treated with CD18 MAb and exposed to ozone, there were no neutrophils, but there was a compensatory increase in alveolar macrophages on the apical surface of the respiratory bronchiolar epithelium that was significantly greater than that in filtered-air control monkeys (Fig. 2). This was in contrast to ozone-exposed monkeys without Ab where neutrophils were the predominant inflammatory cell present in the epithelium. In neutrophil-deficient rats exposed to ozone (8 h at 1 ppm) and given BrdU intraperitoneally, the acute inflammatory response to epithelial injury shows no neutrophil or monocyte recruitment but shows a marked BrdU labeling of alveolar macrophages 48 h after ozone exposure (Hyde, unpublished observations). These data imply that the increase in macrophages at 24 h in CD18 MAb-treated monkeys is the result of in situ alveolar macrophage proliferation. It is of interest that even though there were more alveolar macrophages in CD18 MAb-treated, ozone-exposed monkeys than neutrophils in ozone-exposed monkeys without Ab treatment at 24 h, necrotic epithelial cells remained significantly elevated up to 48 h after ozone exposure (Fig. 3). In situ versus monocyte-derived alveolar macrophage phenotypes may explain, in part, the differences in the removal of injured epithelial cells and subsequent repair of the epithelium by 48 h after ozone exposure (11), but neutrophils clearly play a critical role in this monkey model of ozone-induced epithelial injury.

C5a instillation into the right middle and caudal lung lobes of CD18 MAb-treated monkeys was used to further test this concept. There was a significant decrease in the ethidium-positive cell (necrosis) score in respiratory bronchioles of the right middle lobes (received C5a instillation) compared with that in the left cranial lung lobes of CD 18 MAb-treated, ozone-exposed monkeys (Fig. 9). A similar difference, although not significant, was evident in the ethidium-positive cell score between respiratory bronchioles of the right middle and left cranial lung lobes in monkeys treated with the isotype control. Proximal bronchi showed trends similar to those in the respiratory bronchioles, but there were no significant differences between lobes or groups. These results show that neutrophils play a beneficial role in removing injured epithelial cells. It is also apparent that removal of injured epithelial cells and airway debris by neutrophil recruitment played a beneficial role in epithelial restoration of the monkey respiratory bronchiole (Fig. 8).

With the assumption that neutrophils responding to C5a instillation used the same emigration pathway that has been observed in rabbits, that is, through the capillaries of the interalveolar septa but not through the vessels in airway walls (7), then neutrophils migrated from the alveolar parenchyma to the more proximal respiratory bronchioles to remove injured epithelial cells during the 4 h after ozone exposure in CD18 MAb-treated monkeys. However, we cannot rule out some CD18-independent neutrophil migration through vessels in the airway walls even though we did not observe any neutrophils in transit through the respiratory bronchiolar walls at necropsy because the migration could have occurred subsequent to C5a instillation but before necropsy. No matter what their site of emigration, the possibility exists that CD18-independent neutrophils represent the more effective population in removal of injured epithelial cells. It is not a surprise that neutrophils can remove injured epithelial cells from the airway lumen because Cheek et al. (4) have observed this phenomenon in alveolar epithelial cells in culture after ozone exposure and the addition of neutrophils to the apical side of the epithelium. In endothelial-neutrophil interactions in vitro, the CD18 MAb IB4 showed protective effects predominantly via its antiadherence property because it did not effectively block opsonized zymosan or phorbol 12-myristate 13-acetate-stimulated superoxide anion or myeloperoxidase release (16). Furthermore, the leukocyte response integrin that recognizes the basement membrane protein entactin can act together as a single signal transduction unit to activate the phagocyte respiratory burst independent of CD18 (26). Together, these data suggest
that neutrophils do not require CD18 to kill and/or remove injured epithelial cells.

In conclusion, our findings in rhesus monkeys support the hypothesis that emigration of neutrophils to the site of injury facilitates the repair process by assisting with the destruction and/or removal of ozone-injured airway epithelial cells. We believe that these data in rhesus monkeys show that neutrophils accelerate the onset of tissue repair by removal of ozone-injured epithelial cells.

We thank Dr. Nancy Tyler for preparation of the figures and editing the manuscript and Brian Tarkington for expert technical assistance in the ozone exposure system.

This study was supported by National Institute of Environmental Health Sciences Grant ES-00628, National Center for Research Resources Grant RR-00169, and Tobacco Related Disease Research Program Grant 6kt-0411.

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Received 4 February 1999; accepted in final form 19 July 1999.

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