Importance of Impairment of the Airway Epithelium for Ozone-Induced Airway Hyperresponsiveness in Guinea Pigs

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ABSTRACT—We examined the relationship between ozone (O_3)-induced airway hyperresponsiveness (AHR) and inflammation in guinea pigs. Inhalation of methacholine (MCh) was adopted in the time course study of AHR that was assessed by measuring pulmonary inflation pressure after O_3 exposure (3 ppm, for 2 hr) because the degree of AHR detected by inhalation of MCh was greater than that detected by i.v. administration. AHR was detected up to 5 hr after O_3 exposure and was not observed at 24 and 48 hr. In the bronchoalveolar lavage (BAL) study, the numbers of neutrophils, eosinophils, lymphocytes and macrophages in BAL fluid (BALF) reached maximum at 24 hr or later. On the other hand, the number of airway epithelial cells in the BALF significantly increased at 2 and 5 hr. In the histological study, disorder and impairment of the airway epithelium in the trachea and lung were observed at 2 and 5 hr. Changes in the airway epithelium were recovered at 48 hr, although an increase in leukocytes was observed in the lung. These results indicate that O_3-induced AHR in guinea pigs is most probably associated with impairment of the epithelium rather than with infiltration of inflammatory cells in the airway.

Keywords: Ozone, Airway hyperresponsiveness, Inflammatory cell, Epithelial cell

Airway hyperresponsiveness (AHR) is an important pathophysiological feature of asthma. Accordingly, it is very essential to elucidate the process of AHR and to find a way to improve it. To date, allergen (1, 2)-, virus (3, 4)-, Sephadex beads (5, 6)-, platelet activating factor (7, 8)-, endotoxin (9)- and air pollutant (10)-induced AHR models in various animals are widely used by many laboratories to clarify the mechanism of AHR. However, it has not been elucidated in detail bacause it is a very complicated process.

It is well known that ozone (O_3), an air pollutant, and exposure to humans (11), dogs (12), rats (13), guinea pigs (14) and other animals can cause transient AHR and airway inflammation. Therefore, O_3-induced AHR is used as a tool for investigating AHR. It is said that O_3-induced AHR is due to 5-lipoxygenase metabolites (15), endogenous tachykinin (16) and other factors. On the other hand, some reports indicated that infiltration of neutrophils into the airway lumen was associated with O_3-induced AHR (17-19), but another reports have denied this (13, 20).

In this study, we examined the effect of inflammatory cell infiltration and morphological change in the airway mucosal layer on O_3-induced AHR in guinea pigs using bronchoalveolar lavage (BAL) and histological study in the airway.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs (Japan SLC, Inc., Hamamatsu), weighing 330 to 740 g, were used. These animals were housed in an environmentally controlled room (temperature, 23±2℃; humidity, 55±5%; illumination time, from 7:00 to 19:00) with food and water available ad libitum for 1 week prior to the experiment.

Chemicals

The following chemicals were used: pentobarbital sodium (Abbott, North Chicago, IL, USA), gallamine triethiodide and HEPES (Sigma, St. Louis, MO, USA), methacholine chrolide (MCh; Nacalai Tesque Co., Ltd., Kyoto), Hanks (Nissui Pharmaceutical Co., Ltd., Tokyo), heparin (Mochida Pharmaceutical Co., Ltd.,

Tokyo), Giemsa's stain solution (Merck, Rahway, NJ, USA). These reagents were laboratory grade or better.

O_3 exposure

Animals in an acryl chamber (29 × 19 × 25 cm) inhaled O_3 produced by an O_3 generator (OZX-02; Silver Seikou, Tokyo) for 2 hr. During O_3 exposure, it was closely monitored by an O_3 detector (SOZ-3000 and -3100; Seki Electronics, Tokyo) to keep its concentration at 3 ppm. Animals in the control group not exposed to O_3 were placed in cages until they were anesthetized for measuring airway reactivity.

Measuring of airway reactivity

The O_3-exposed and control animals were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and artificially ventilated by a Harvard 683 respirator (10 ml/kg, 60 strokes/min) through a tracheal cannula. Spontaneous respiration was stopped by gallamine triethiodide (5 mg/kg, i.v.). Pulmonary inflation pressure (PIP) as the index of airway reactivity was measured by a pressure transducer (LPU-0.1; Nihon Kohden, Tokyo) connected to a tracheal cannula.

Administration of spasmogen

To assess O_3-induced AHR, the animals were challenged by MCh, as a spasmogen, through i.v. administration (0.3 − 10 μg/kg) via the lateral saphenous vein or by inhalation. On inhalation challenge of MCh, the tracheal cannula connected to a container (29 φ × 50 mm) that contained 5 ml of MCh saline solution (0.1 − 1000 μg/ml); and under the above condition of respiration, aerosol was generated with an ultrasonic nebulizer (TUR-3000, Nihon Kohden) for 2 min.

The index of AHR

The provocative dose and the concentration of MCh that caused a 5 cmH_2O increase in PIP (ΔPIP) was expressed as PD_5 by i.v. administration and as PC_5 by inhalation. As the index of AHR, $logPD_5$ and $logPC_5$ values were determined.

Bronchoalveolar lavage (BAL)

Animals were sacrificed by means of intraperitoneal injection of pentobarbital sodium (100 mg/kg, i.p.) 2, 5, 24, 48 and 192 hr after O_3 exposure. The trachea was immediately cannulated, and bronchoalveolar lavage fluid (BALF) was obtained by injection of 10 U/ml heparin- and 25 mM HEPES-containing sterile Hanks solution (pH 7.2, volume: 5 ml × 5/animal, Hanks(+)) warmed to 37℃ into the trachea. The obtained BALF was centrifuged at 400 × *g* for 5 min at 4℃. The obtained precipitate was suspended to 1 ml/animal of Hanks(+), and the number of total cells in 20 μl of suspension was counted by a Sysmex Platelet Counter (PL-110; Toh-a Medical Electronics, Co., Ltd., Kobe). A 20-μl aliquot of the cell suspension was used for a differential count that was made on a smear prepared and stained by Giemsa's stain solution. The numbers of neutrophils, eosinophils, lymphocytes, macrophages and epithelial cells were counted by a light microscope at a magnification × 1,000. Airway epithelial cells were discriminated by recognition of the cobblestone appearance typical of epithelial cells in light microscopic examination.

Histological study

The animals were anesthetized with pentobarbital sodium (30 mg/kg, i.p.), and were transcardially perfused with 10% phosphate-buffered formalin (PBF) for 5 min. The trachea and lung were then isolated from control and O_3-exposed animals 2, 5 and 48 hr after O_3 exposure. Isolated trachea and lung were fixed in 10% PBF, embedded in paraffin, cut and stained with hematoxylin-eosin, and they were observed using a light microscope.

Statistical analyses

All results are expressed as the mean ± S.E.M. except for the data from the histological study. Comparisons of the mean values were made by Student's *t*-test or analysis of variance followed by the Tukey-Kramer's method.

RESULTS

Assessment of O_3-induced AHR by i.v. administration of MCh

The airway reactivities in air (control)- or O_3-exposed guinea pigs were investigated by i.v. administration of MCh 2 hr after air or O_3 exposure, and the results are shown in Fig. 1 and Table 1. I.v. administration of MCh dose-dependently elevated PIP from 3 μg/kg, i.v. in the control. The O_3 exposure tended to produce a leftward shift in the MCh dose-response curves (Fig. 1), while $logPD_5$ values were not significantly lower compared with the control (Table 1).

Assessment of O_3-induced AHR by inhalation of MCh

The airway reactivities in air (control)- or O_3-exposed guinea pigs were investigated by inhalation of MCh, and the results are shown in Fig. 2 and Table 1. Inhalation of MCh concentration-dependently increased PIP from 30 μg/ml, 2 min inhalation in the control. O_3 exposure produced a significant leftward shift in the MCh concentration-response curves (Fig. 2). $LogPC_5$ in the O_3-exposed groups were significantly lower compared with the control (Table 1).

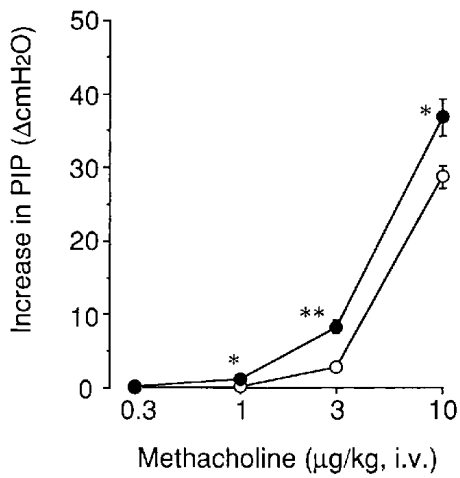

Fig. 1. Dose-response curves for i.v. methacholine-induced bronchoconstriction in air (○, control)- or O_3 (●)-exposed guinea pigs. Guinea pigs inhaled air or 3 ppm of O_3 for 2 hr. Airway reactivities were measured 2 hr after air or O_3 exposure. Each point represents the mean±S.E.M. of 4 experiments. *P<0.05, **P<0.01, statistically significant compared with the control group by Student's *t*-test.

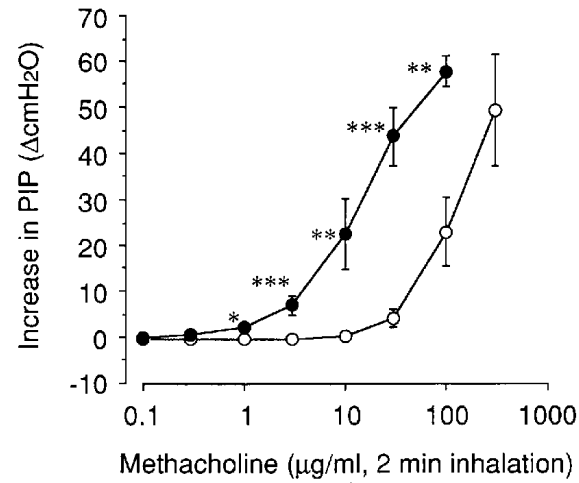

Fig. 2. Concentration-response curves for methacholine aerosol-induced bronchoconstriction in air (○, control)- or O_3 (●)-exposed guinea pigs. Guinea pigs inhaled air or 3 ppm of O_3 for 2 hr. Airway reactivities were measured 2 hr after air or O_3 exposure. Each point represents the mean±S.E.M. of 4 experiments. *P<0.05, **P<0.01, ***P<0.001, statistically significant compared with the control group by Student's *t*-test.

Table 1. Airway reactivity to i.v. administration or inhalation of methacholine in air- and O_3-exposed guinea pigs

	O_3	
	− (air)	+
$logPD_5$ (i.v.)	3.52±0.01	3.27±0.04
$logPC_5$ (inhalation)	4.49±0.13	3.31±0.17**

Animals inhaled 3 ppm of O_3 for 2 hr. Pulmonary inflation pressure (PIP) was used as the index of bronchoconstriction. PD_5 and PC_5 are defined as the provicative dose (ng/kg, i.v.) and provocative concentration (ng/ml, 2 min inhalation) of methacholine that increased PIP by 5 cmH_2O, respectively. Each value represents the mean±S.E.M. of 4 experiments. **P<0.01, satistically significant compared with the O_3(−) group by Student's *t*-test.

Time course of AHR after O_3 exposure

To investigate the persistence of AHR caused by 3 ppm of O_3 exposure for 2 hr, the time course of airway reactivity to inhalation of MCh after O_3 exposure was studied, and the results are shown in Fig. 3 and Table 2. The concentration-response curve to MCh was significantly shifted to the left at 2 and 5 hr, but not at 24 and 48 hr (Fig. 3). The values of $logPC_5$ in O_3-exposed guinea pigs were also significantly lower at 2 and 5 hr, but not at 24 and 48 hr (Table 2), compared with the control. Namely, these data indicated that O_3-induced AHR was diminished from 5 to 24 hr.

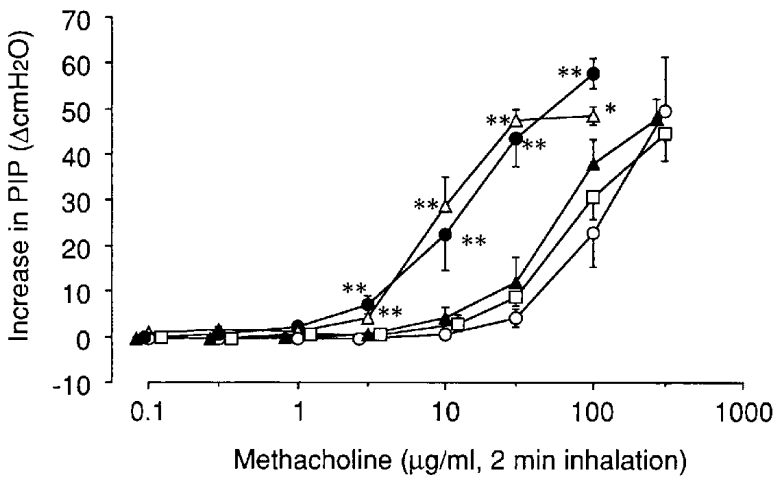

Fig. 3. Time course after O_3 exposure of concentration-response curves for methacholine-induced bronchoconstriction. Guinea pigs inhaled air or 3 ppm of O_3 for 2 hr. Methacholine aerosol-induced bronchoconstriction was measured in the control (○) and 2 (●), 5 (△), 24 (▲) and 48 (□) hr after O_3 exposure. Each point represents the mean±S.E.M. of 4−6 experiments. *P<0.05, **P<0.01, statistically significant compared with the control group by one-way ANOVA (Tukey-Kramer's method).

Table 2. Time course of airway reactivity to methacholine aerosol in guinea pigs after O_3 exposure

Time after O_3 exposure	$logPC_5$ (ng/ml, 2 min inhalation)
Control	4.56±0.13
2 hr	3.31±0.17**
5 hr	3.45±0.09**
24 hr	4.15±0.21
48 hr	4.17±0.16

Animals inhaled 3 ppm of O_3 for 2 hr. Pulmonary inflation pressure (PIP) was used as the index of bronchoconstriction. PC_5 is defined as the provocative concentration (ng/ml, 2 min inhalation) of methacholine that increased PIP by 5 cmH_2O. Each value represents the mean±S.E.M. of 4–8 experiments. **$P<0.01$, satistically significant compared with the control group by one-way ANOVA (Tukey-Kramer's method).

BAL after O_3 exposure

The results of the BAL study are shown in Figs. 4 and 5. The cells recovered in the BALF were classified into neutrophils, eosinophils, lymphocytes, macrophages and epithelial cells; and the number of cells in each cell population was counted under a light microscope following staining. The number of neutrophils slightly increased 24 hr after O_3 exposure, but this was not significant. The number of eosinophils and lymphocytes at 48 hr was $26.3(\pm5.8)\times10^5$ and $3.4(\pm0.6)\times10^5$ cells/BALF/animal, respectively. These were significant increases compared with the control ($6.7(\pm4.3)\times10^5$ and $0.4(\pm0.1)\times10^5$ cells/BALF/animal, respectively). Macrophages were increased from 24 hr, reaching a maximum at 96 hr ($110.6(\pm17.6)\times10^5$ cells/BALF/animal). This increase was recovered at 192 hr to the control level. Significant increases in epithelial cells compared with the control ($0.9(\pm0.2)\times10^5$ cells/BALF/animal) were recognized at 2 and 5 hr ($7.0(\pm2.0)\times10^5$ and $15.1(\pm3.4)\times10^5$ cells/BALF/animal, respectively), but epithelial cells did not significantly increase after 24 hr and later on.

Histological study in central and peripheral airway

In the BAL study, epithelial cells in the BALF significantly increased 2 and 5 hr after O_3 exposure when AHR was detected. Accordingly, the preparations of the trachea and the lung at 2, 5 and 48 hr and in the control were observed under a light microscope, and the results are shown in Figs. 6 and 7. Shedding, vacuolation and derangement of the epithelium in the trachea and the lung

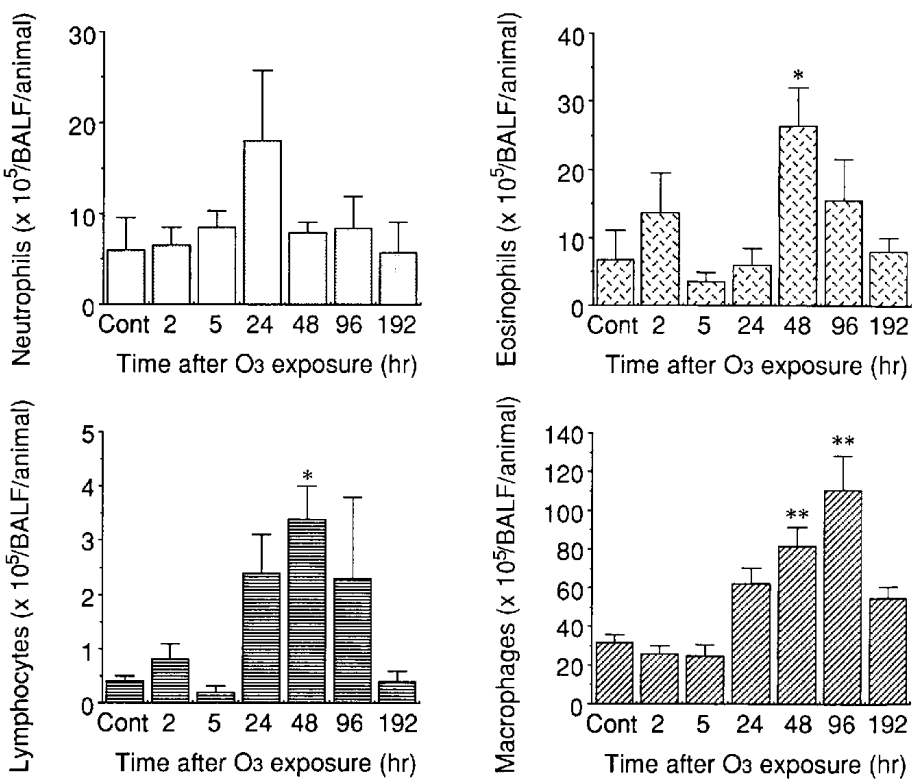

Fig. 4. Changes in the numbers of neutrophils, eosinophils, lymphocytes and macrophages in BALF after O_3 exposure in guinea pigs. Each column represents the mean±S.E.M. of 7 experiments. *$P<0.05$, **$P<0.01$, statistically significant compared with the control group by one-way ANOVA (Tukey-Kramer's method).

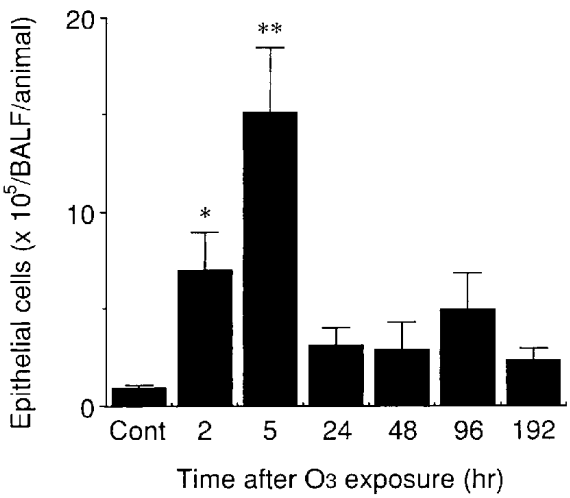

Fig. 5. Changes in the number of airway epithelial cells in BALF after O_3 exposure in guinea pigs. Each column represents the mean±S.E.M. of 7 experiments. *$P<0.05$, **$P<0.01$, statistically significant compared with the control group by one-way ANOVA (Tukey-Kramer's method).

were commonly recognized at 2 and 5 hr (Figs. 6b, 6c, 7b and 7c), whereas the epithelium was recovered at 48 hr (Figs. 6d and 7d). On the other hand, an increase in leukocytes in the alveolar septa and alveolar ducts in the lung was recognized at 48 hr (Fig. 7d), but not in the control and at 2 and 5 hr (Figs. 7a, 7b and 7c).

DISCUSSION

Some researchers (17–19) have emphasized the association between O_3-induced AHR and infiltration of inflammatory cells, especially neutrophils. Weideman and Schlesinger (21) reported that neutrophils in humans and rabbits were activated to change eicosanoid metabolism following O_3 exposure in vitro. However there have been some arguments against the involvement of inflammatory cells in O_3-induced AHR (13, 20). Accordingly, it remained necessary to investigate how inflammatory cells were associated with O_3-induced AHR. In this study, we assessed the transient AHR observed by administration of MCh after 3 ppm of O_3 exposure for 2 hr and investigated the association between O_3-induced AHR and airway inflammation.

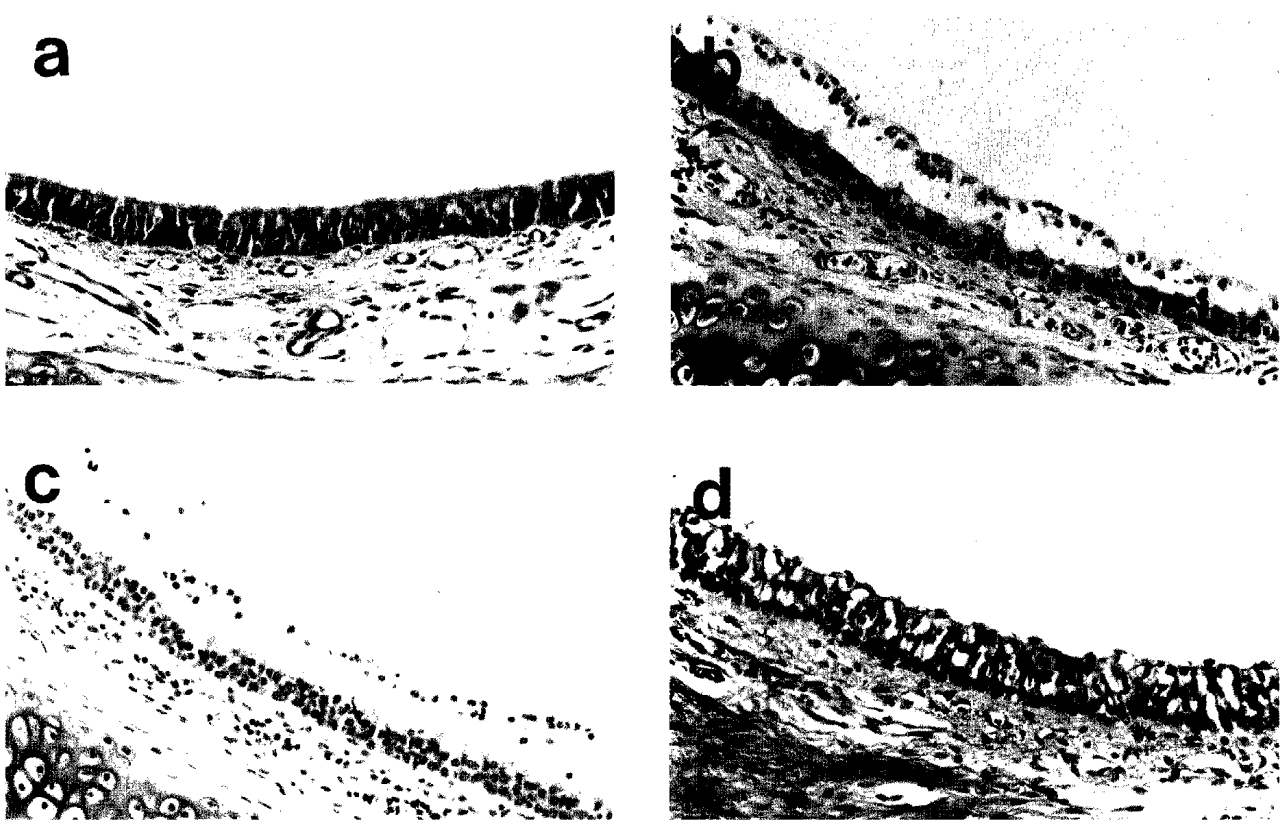

Fig. 6. Representative photographs of tracheal mucosa in air- and O_3-exposed guinea pigs. a: Air-exposed (control), b: 2, c: 5 and d: 48 hr after O_3 exposure. Derangement and impairment of the tracheal epithelium were recognized in b and c. The recovery of the epithelium was observed in d. Magnification ×150.

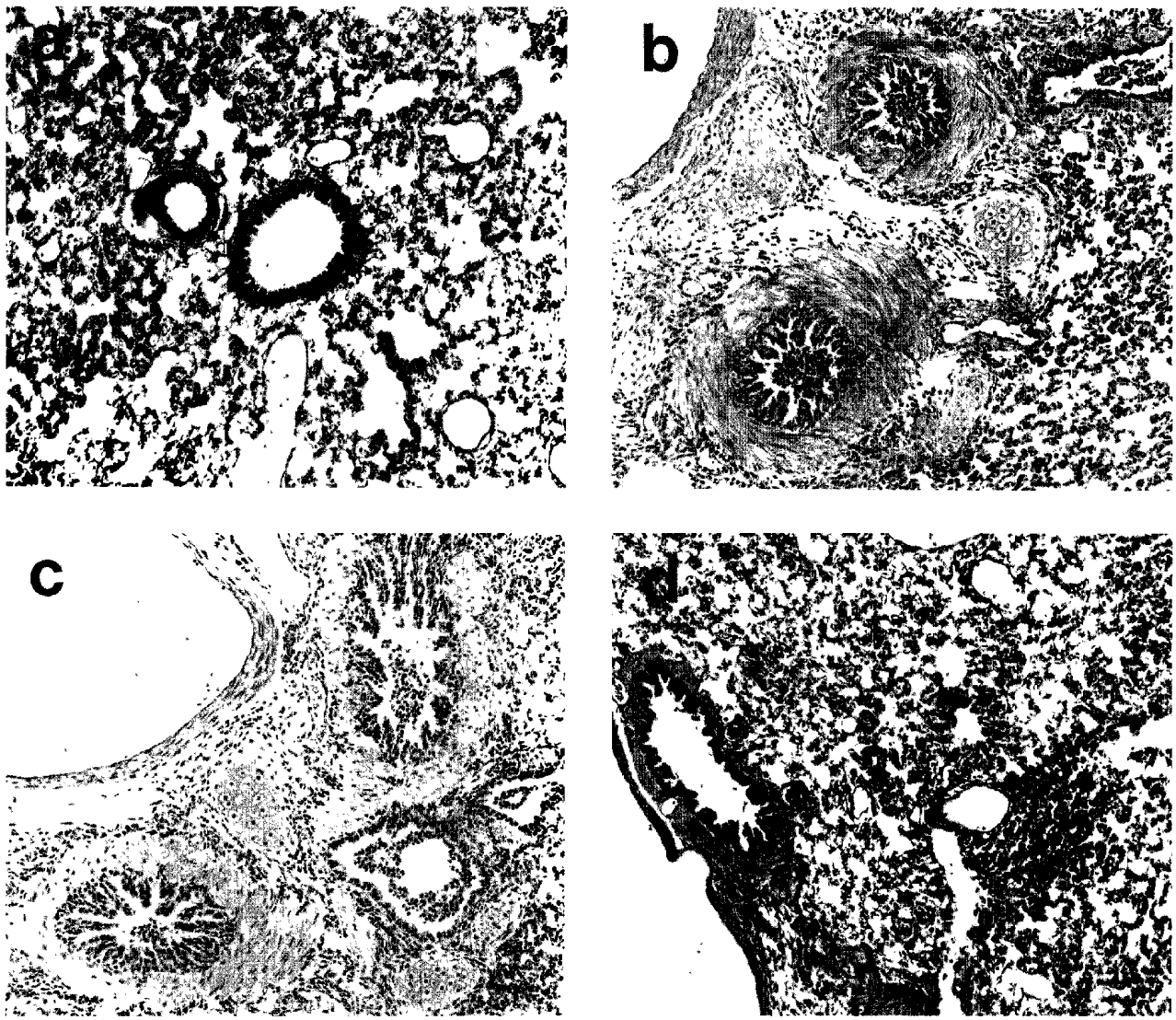

Fig. 7. Representative photographs of peripheral airway in the lung in air- and O_3-exposed guinea pigs. a: Air-exposed (control), b: 2, c: 5 and d: 48 hr after O_3 exposure. Derangement and impairment of the epithelium were recognized in b and c. The recovery of the epithelium was observed in d. An increase in leukocytes was recognized in d, but not in a, b and c. Magnification ×75.

Dose- or concentration-response curves to MCh were shifted to the left by O_3 exposure (Figs. 1 and 2). The degree of AHR from the values of $logPD_5$ and $logPC_5$, however, was detected by inhalation of MCh but not by i.v. administration (Table 1). Yeadon and Payne (22) reported that inhalation of spasmogen was available for AHR detection but not i.v. administration in guinea pigs exposed to 3 ppm of O_3 for 30 min. Therefore, it is conceivable that O_3 acted at the airway luminal side. Since then, we adopted inhalation of MCh to detect AHR, although AHR has been assessed by i.v. administration of spasmogens in some laboratories.

It is well understood that O_3-induced AHR and inflammation are transient (23). Because some reports suggested that O_3-induced AHR is associated with infiltration of inflammatory cells including neutrophils in the airway (17–19), we first examined the time course of AHR after O_3 exposure. In this study, the AHR was observed up to 5 hr, and it was already diminished from 24 hr (Fig. 3 and Table 2). At the same time, the time course study of changes in the numbers of infiltrated inflammatory cells was performed by BAL. Infiltration of inflammatory cells was not recognized up to 5 hr when AHR was detected (Fig. 4), and it increased or reached a maximum at 24 hr or later when AHR was already diminished. However, the number of epithelial cells in the BALF significantly in-

creased up to 5 hr when AHR was detected (Fig. 5). In the histological study, the preparations of the trachea and lung were observed by a light microscope (Figs. 6 and 7). Disorder and impairment of epithelial cells were observed, and inflammatory cells were not recognized in the mucosal and submucosal layers at 2 and 5 hr. In the preparations at 48 hr, the epithelium was recovered in the trachea and lung, and inflammatory cells in the lung increased. These results indicating the conflict between O_3-induced AHR and increase in inflammatory cells in the airway are consistent with the results of Evans et al. (13) who used rats, and the impairment of the airway epithelium coincided with the persistence of AHR. As to the role of epithelial cells, they are involved in the metabolism and the breakdown of endogenous and exogenous spasmogens (24), the protection against irritants entering into the airway from one's external environment (24–27), the control of airway smooth muscle contraction (24, 28, 29), excretion of sputa and foreign bodies (24, 30) and so on. On the other hand, it was demonstrated that O_3 injured the lipid layer of the cell membrane by peroxidation in vitro (31). Namely, it is likely that O_3 directly injured the epithelial cells by peroxidation and that AHR appeared following the loss of epithelial cell functions. Therefore, we can suppose that spasmogens fail to be metabolized by epithelial cells and/or easily reach the smooth muscle in the airway tissue in the mechanism of O_3-induced AHR. Furthermore, the active sites (for example, disclosure of C-fiber, etc.) for spasmogens under the mucosal layer may appear simultaneously following the shedding of the epithelium.

Our results indicate that O_3-induced AHR may be caused by loss of normal epithelial cell functions, but not by infiltration of inflammatory cells into the airway. It is said that many mediators are important for O_3-induced AHR (15, 16). Further studies of the origin and the influence of those mediators will be necessary to define the nature of O_3-induced AHR.

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