

# Histopathology of the airway epithelium in an experimental dual-phase model of bronchial asthma

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## Summary

**Background** Lesions of trachea cuticles are a pathological histological characteristic of bronchial asthma. Furthermore, collected tracheal cuticles desquamated from the respiratory tract are found in patients' sputum when asthma attacks occur or after the induction of allergen inhalation. From these facts, it is assumed that desquamation of trachea cuticle cells is a pathological symptom of bronchial asthma. However, there has not been any chronological report of desquamation of trachea cuticles through the process of bronchial asthma attacks.

**Objective** For this report, we made an experimental bronchial asthma model using guinea pigs, and conducted chronological examinations of trachea cuticle lesions related to pathological symptoms of bronchial asthma using a transmission electron microscope and a scanning electron microscope.

**Methods** The experimental asthma models were made by injection of ovalbumin into the abdominal cavity of guinea pigs. Then the airway responses to inhaled aerosolized ovalbumin were induced. The trachea were enucleated and examined under an optical microscope, a transmission electron microscope (hereafter abbreviated as TEM), and a scanning electron microscope (hereafter abbreviated as SEM) after 1, 2, 4, 8, 12, 24 h and 7 days after the immediate airway responses.

**Results** Intercellular oedema of ciliated epithelium was observed in the sensitization groups immediately after the immediate airway response. SEM observation revealed increased mucus secretion and shortening of cilium. A slight case of desquamation or deciduation of ciliated epithelium was also beginning to appear. TEM observation revealed a dilation of ciliated epithelium intervals. Infiltration of eosinophilic leucocytes was already detectable beneath the ciliated epithelium. The degree of ciliated epithelium desquamation and infiltration of eosinophilic leucocytes progressed with time. When the late airway response occurred 4 hours later, eosinophilic leucocytes had increased drastically, and ciliate epithelium had desquamated to the extent that basal cells were exposed. Seven days after the immediate airway response, epithelium intercellular oedema had improved, and cilium had been reproduced.

**Conclusion** These results suggest that desquamation of epithelium caused by trachea cuticle lesions appears at an early stage of an asthma attack, owing to the contraction of the trachea, and that the damage is intensified by the infiltration of eosinophilic leucocytes.

**Keywords:** ciliary injury, immediate asthmatic response, late asthmatic response, experimental asthma, major basic protein, scanning electron microscope, transmission electron microscope.

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## Introduction

One of the characteristic conditions of bronchial asthma is inflammation in the airway epithelium, and a close relationship between inflammation in the airway epithelium and enhancement of bronchial sensitivity has been reported [1]. This correlation is probably due to the responses of the sensory nerve terminal irritant receptors and c-fibers disclosed by the disruption of airway epithelia due to inflammation [2] caused by stimuli. This results in enhancement of bronchial sensitivity by vagovagal reflex [3] and axon reflex [4], increased penetration of antigens and substances that induce airway contraction through the disrupted epithelium [5], stenosis, and sensitivity of the airway to various inflammatory mediators released by inflammatory cells that have migrated into the bronchial tissues. The increases in the permeability of the airway caused by the disruption of the airway epithelium and lack of the epithelial-derived relaxing factor (EpDRF) and neuroendopeptidase (NEP) released from the airway epithelium are also considered to induce contraction of the airway [4,6–8]. Thus, it is important to clarify whether bronchial asthma causes disruption and deciliation of the airway epithelium.

It has been shown that clusters of columnar cells from the airway epithelium, which were named Creola bodies (CrB) by Naylor, are observed in the sputum of patients with asthma [9]. Some other reports also suggest that disruption of the airway epithelium occurs, based on the observations of the airway epithelium in the sputum during a bronchial asthma attack or after an inhaled antigen challenge [10–12]. However, it has not been confirmed that disruption of the ciliated epithelium in the airway is caused by the bronchial asthma attack. In this study, inflammation in the airway epithelium in bronchial asthma was electron-microscopically examined by focusing on disruption of the airway epithelium, using a guinea pig model with dual-phase bronchial responses.

## Methods

### *Experimental animals*

The animals were male Hartley guinea pigs. Untreated animals comprised the control group and animals sensitized by intraperitoneal injection of ovalbumin (OA, Grade V, SIGMA Chemical Co., MO, USA) comprised the sensitized group.

### *Sensitization method*

According to the method developed by Tohda [13], guinea pigs weighing 250–350 g were pre-treated with 30 mg/kg of cyclophosphamide (Shionogi Pharmaceutical, Osaka,

Japan) administered by intraperitoneal injection, and 2 days later they were sensitized to OA with an intraperitoneal injection of 1000 µg of OA and 100 mg of Al(OH)<sub>3</sub> (Wako Pure Chemicals, Osaka, Japan). Three weeks later, they were given a booster injection of 10 µg of OA and 100 mg of Al(OH)<sub>3</sub>. The animals in the control group were not given any of the above treatments.

### *Method of confirming airway reaction to inhaled OA*

Three weeks after the completion of sensitization, animals were placed in a body plethysmograph for small animals and challenged with aerosolized OA (2 mg/mL) using a nebulizer (Devilbiss, Type 646, Pennsylvania, USA) for 1 min. The ventilation per unit of time (VT) was measured before, as well as 1, 3 and 5 min after the challenge to confirm OA-induced airway allergic responses. If VT was reduced more than 20% compared to VT before inhalation, asthmatic airway reaction was considered positive. Airway reactions observed immediately after inhalation were

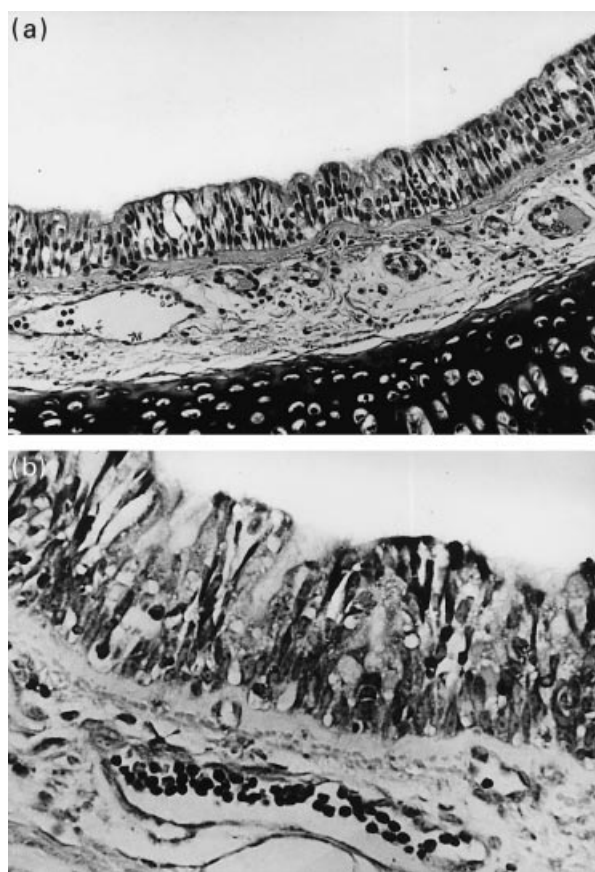


**Fig. 1** (a) Control group (non-sensitized): no disruption of ciliated cells was observed under light microscopy (HE staining  $\times 200$ ). (b) Sensitized no-challenge group: eosinophils were present in blood vessels (May-Gruenwald-Giemsa staining  $\times 400$ ).

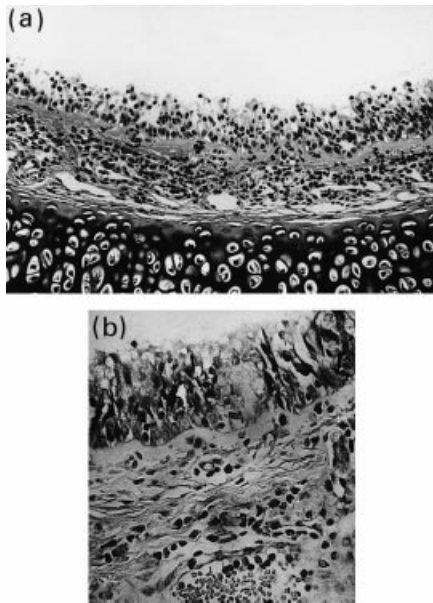


**Fig. 2.** IAR group: (a) oedema was observed in the ciliated epithelial cells and inflammatory cells had begun to infiltrate (HE staining  $\times 200$ ). (b) A slight increase in infiltration of eosinophils from the blood vessels in tissue and ciliated epithelial cells (May-Gruenwald-Giemsa staining  $\times 400$ ).

regarded as immediate asthmatic responses (IAR), and those observed after the disappearance of IAR were regarded as late asthmatic responses (LAR).

#### Examination protocol

The examinations were performed in the control and sensitized groups as follows:

- non-sensitized;
- sensitized, no-challenge; and
- sensitized with attack (IAR, 1, 2, 4 (LAR), 8, 12, 24, 48 h and 7 days after the IAR).

#### Preparation of samples

**Preparation of samples for microscopy** The animals were anaesthetized by intraperitoneal injection of 50 mg/kg of pentobarbital. After tracheotomizing the superior larynx, a probe was inserted, and 3% glutaraldehyde was injected at a pressure adjusted to the pressure of the airway. The airway and lung were removed *en bloc*, and a 1-cm long sample of the airway was taken from the bifurcation of the airway towards the mouth. In order to remove mucus the collected samples were thoroughly rinsed with jet streams of physiological saline.

**Light microscopy** The collected samples were immediately immersed in formalin, stained with haematoxylin-eosin (HE)

stain and May-Gruenwald-Giemsa stain, and then observed under the light microscope.

**Scanning electron microscopy (SEM)** The collected samples were immediately rinsed with jet streams of physiological saline to remove mucus, followed by serial fixation with 3% glutaraldehyde (0.05 M cacodylate buffer, pH 7.4), and 1.5% osmium tetroxide. Then they were dehydrated in a graded series of alcohol, immersed in amylacetate, and dried with liquid carbon dioxide at the critical point. The dried samples were fixed on the stage under a dissecting microscope, subjected to ion-sputtering coating with Au-Pd, and observed using a scanning electron microscope (JEOL, JSM-840, Tokyo, Japan).

**Transmission electron microscopy (TEM)** The samples obtained using the above method were double-fixed with 3% glutaraldehyde and 1.5% osmium tetroxide, and dehydrated in a graded series of alcohol. Epon embedding was performed by Luft's method, and ultrathin sections were produced using an ultramicrotome (8800 Ultratome R III, LKB-produkter AB, Bromma, Sweden). The ultrathin sections were placed on a copper grid, double-stained with uranium acetate and lead citrate, then observed using a transmission electron microscope (HS-9 and HU-12 A, Hitachi Ltd, Ibaragi, Japan).

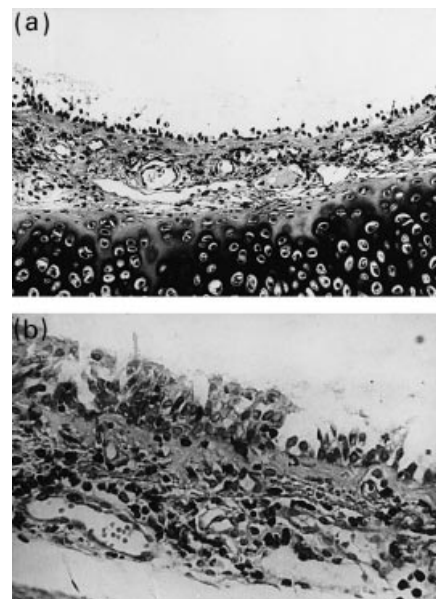


**Fig. 3.** LAR group: (a) decidualization of ciliated epithelial cells caused by marked disruption of ciliated cells and infiltration of lymphocytes was observed (HE staining  $\times 200$ ). (b) Specific eosinophil granules released by eosinophils that had migrated out of side blood vessels were degranulated in the tissue (May-Gruenwald-Giemsa staining  $\times 400$ ).

### Examination of inflammation in the airway epithelium induced by a major basic protein (MBP)

**Preparation of airway rings** The animals were anaesthetized by intraperitoneal injection of 50 mg/kg of pentobarbital, and the airway was removed under sterile conditions. The samples were sliced into 1-mm long sections, rinsed in Eagle's MEM containing penicillin and streptomycin, and incubated on Falcon multiwell tissue culture plates with 1 ml/well Eagle's MEM at 37 °C, 95% humidity, and 5% CO<sub>2</sub> concentration for 3 days before being used in experiments.

**Incubation with MBP** The airway rings placed on Falcon multiwell tissue culture plates with 1 ml/well Eagle's MEM were incubated with 10, 100 or 500 µg/mL of MBP (provided by Prof. G.J. Gleich, Mayo Clinic, MN, USA) for 5 min, 4 h or 48 h, then observed by scanning and transmission electron microscopy.

### Statistical analysis

The data are presented as the mean  $\pm$  SD. Statistical comparisons were performed using Student's *t*-test. Statistical significance was accepted at the 5% level of confidence.

## Results

### Light microscopic observation

In non-sensitized (control, Fig. 1, a) and sensitized no-challenge (Fig. 1, b) groups, no disruption of ciliated cells caused by oedema, such as cellular fluid in the ciliated epithelium, was observed under light microscopy. Eosinophils were present in blood vessels, but no infiltration was noted in the airway epithelium, showing no difference in the infiltration of inflammatory cells, eosinophils in particular, in the airway epithelium between the two groups. On the other hand, oedema in the ciliated epithelial cells was observed in the samples treated by HE staining, at the time IAR was induced by an OA inhalation challenge in the sensitized animals, and inflammatory cells (Fig. 2, a) had begun to infiltrate.

The samples treated by May-Gruenwald-Giemsa staining showed a slight increase in infiltration of eosinophils in the blood vessels, tissues and ciliated epithelial cells (Fig. 2, b). In the samples at the time of LAR, exfoliation of basal cells and infiltration of lymphocytes caused by marked disruption and decudation of ciliated epithelial cells was observed (Fig. 3, a), and specific eosinophil granules released by eosinophils migrating outside of blood vessels were degranulated in the tissues (Fig. 3, b). Time course of eosinophil infiltration after antigen challenge is shown in

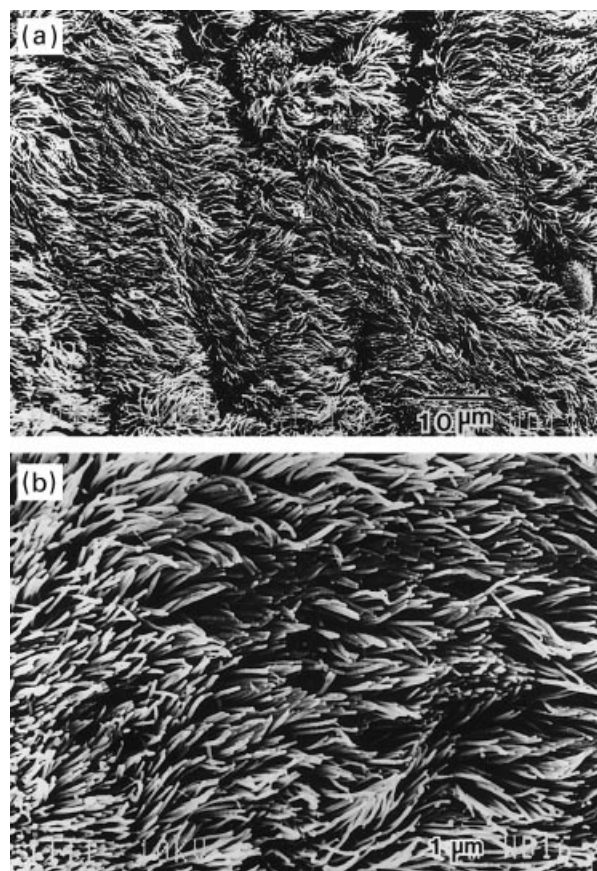


**Fig. 4.** (a) Control group (non-sensitized). (b) Sensitized no-challenge group: the cilia were not histopathologically changed (SEM).

Table 1. Significant infiltration was observed at the time of LAR.

### Scanning electron microscopic observations

In the control (non-sensitized) and sensitized no-attack groups, the cilia were not morphologically changed (Fig. 4, a and b). In the samples at the time of IAR, secreted

**Table 1.** Time course of eosinophil infiltration after antigen challenge

| Group (number of animals) | Number of eosinophils (mean $\pm$ SD) |
|---------------------------|---------------------------------------|
| Control ( <i>n</i> = 3)   | 0.80 $\pm$ 1.0                        |
| IAR ( <i>n</i> = 4)       | 10.41 $\pm$ 3.73                      |
| LAR ( <i>n</i> = 4)       | 77.90 $\pm$ 23.79, <i>P</i> < 0.01    |
| 48 h ( <i>n</i> = 4)      | 23.27 $\pm$ 7.20                      |
| 7 days ( <i>n</i> = 3)    | 1.22 $\pm$ 1.81                       |

10 fields were counted blind at  $\times$  400 magnification.

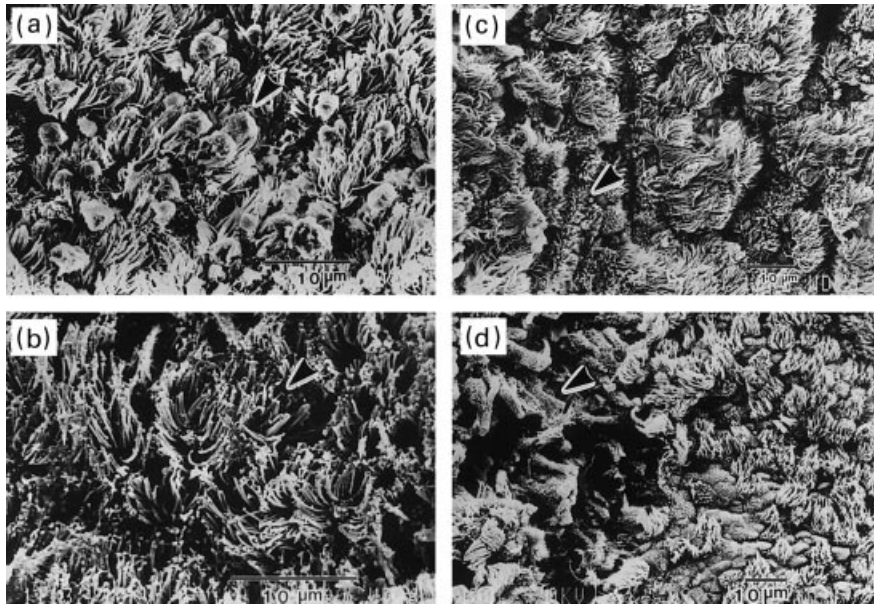


**Fig. 5.** IAR group (SEM): (a) mucus secretion resembles a dome from the mucus gland; (b) enhancement of mucus secretion resulting in formation of tangled threads of the mucus between cilia was observed; (c) shortening cilia; (d) slight disruption and decidualization of ciliated epithelial cells and exfoliation of some of the small mucus granule cells to the lumen were observed.

material resembled a dome from the mucus gland (Fig. 5, a) and enhancement of mucus secretion was observed, resulting in formation of tangled threads of the mucus between cilia (Fig. 5, b). The cilia were shortened (Fig. 5, c, shortening cilia). Slight disruption and decidualization of ciliated epithelial cells and exfoliation of some of the small mucous granule cells (SMG) to the tracheal lumen (Fig. 5, d) were observed.

In the samples at the time of LAR, disruption and decidualization of ciliated epithelial cells were seen over a much larger area than in the samples at the time of IAR (Fig. 6, a). Disorientation of cilia (Fig. 6, b) and further disruption and decidualization of ciliated epithelial cells (Fig. 6, c) shown by bare of basal cells (Fig. 6, d) were noted. Eight hours and 48 h after the occurrence of IAR, not only were there similar changes in the cilia and ciliated

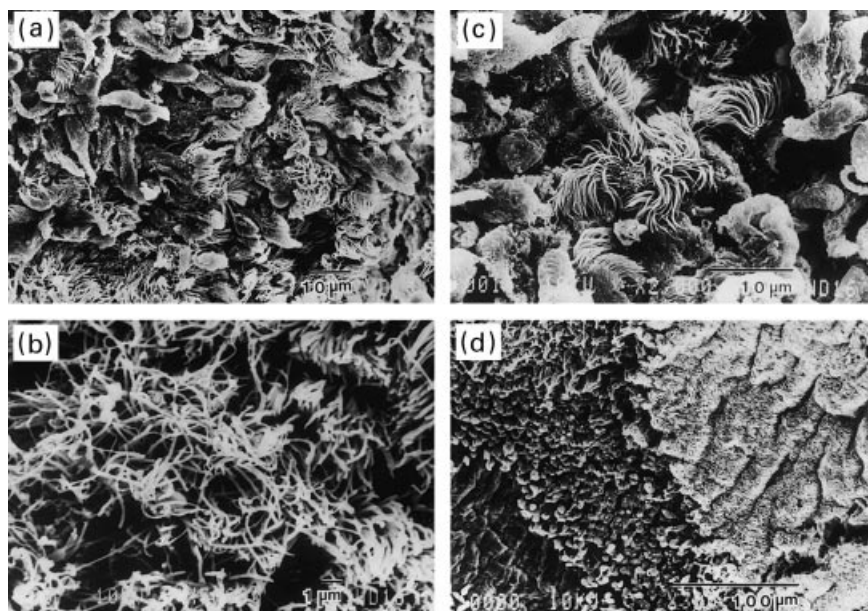


**Fig. 6.** LAR group (SEM): (a) disruption and decidualization of ciliated epithelial cells were observed over a much larger area than at the time of IAR; (b) disorientation of cilia; (c) further disruption and decidualization of ciliated epithelial cells shown by bare of basal cells were noted; (d) bare of basal cells.

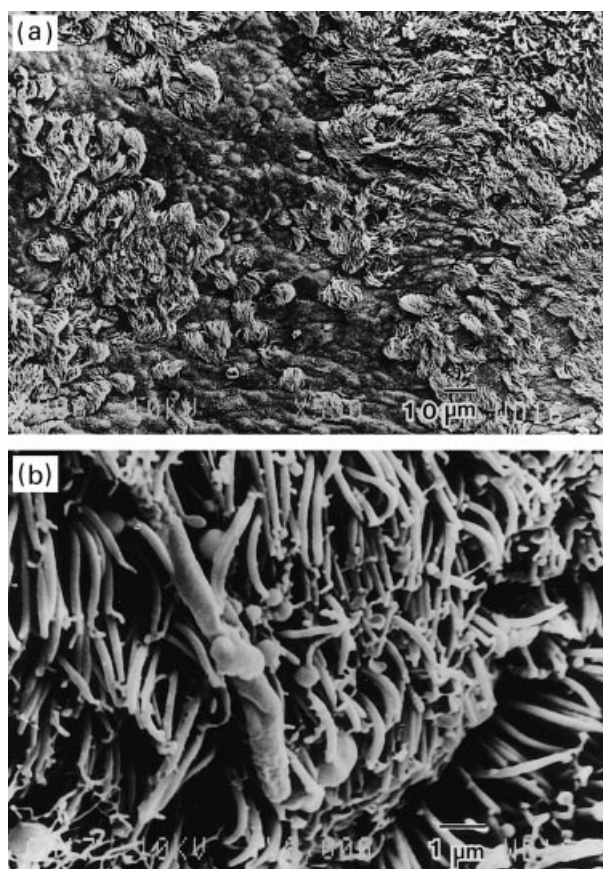


**Fig. 7.** Seven days after IAR (SEM): (a) repair of ciliated cells had begun, showing some regeneration of cilia; (b) unrepaired ciliated epithelial cells and compound cilia were observed in a relatively large area.

epithelial cells, but migration of many eosinophils in the tissues and degranulation were observed. Seven days after the occurrence of IAR, repair of ciliated cells had begun, showing some regeneration of cilia (Fig. 7, a). However, unrepaired ciliated epithelial cells and compound cilia were observed in a relatively large area (Fig. 7, b), suggesting that asthmatic attacks lead to deceduation of ciliated epithelial cells, and that the repair of ciliated epithelial injuries takes more than 7 days.

#### *Transmission electron microscopic observation*

No abnormalities were noted in the control groups (Fig. 8,a). At the time of IAR, rapid bronchial spasm and gaps occurred between ciliated epithelial cells, and eosinophil infiltration was present underneath the epithelium (Fig. 8, b). However, degranulation was not observed in the eosinophils (Fig. 8, c). Upon the occurrence of LAR, further deceduation of ciliated epithelial cells, migration of

many eosinophils from blood vessels, and exfoliation of basal cells were detected (Fig. 8, d).

#### *Changes in the ciliated epithelial cells by MBP*

The airway rings and MBP were incubated and examined by scanning and transmission electron microscopy. There were no changes in the 5-min samples incubated in MBP at all concentrations. In the samples incubated in 100 µg/mL MBP for 24 h, cilia that had been broken off or had attacked lumps of decomposed cilia fusion were observed under scanning electron microscopy (Fig. 9). Deceduation of ciliated epithelial cells was noted, and exfoliation of SMC had begun. After 48 h, further disruption of ciliated epithelial cells and exfoliation of basal cells had occurred.

#### **Discussion**

Asthma is now pathologically regarded as 'chronic desquamation eosinophilic bronchitis' and disruption and deceduation of airway epithelial cells [14,15] are considered to play important roles in the degree of seriousness of asthma [1]. Many studies have suggested that there is a relationship between disruption of airway epithelial cells and bronchial sensitivity. First, disruption of airway epithelial cells by stimuli such as viruses [16,17], chemicals [18] and cigarettes [19] has been confirmed in animal models of bronchial hypersensitivity. Second, disruption of airway epithelial cells has been demonstrated by fiberoptic bronchoscopy in patients with asthma and in their bronchoalveolar lavage fluid. Third, there is a proven correlation between the number of airway epithelial cells in BAL of guinea pigs that have been sensitized to OA and the degree of reduction of the histamine threshold after an inhaled antigen challenge [20]. A positive correlation was also observed between the increase in bronchial sensitivity observed over a 2-week period following vaccination against influenza and the degree of inflammation in airway epithelial cells in dogs [21].

There is growing interest in the role of lymphocytes and eosinophils in asthma. Eosinophils produce a large amount of LTC<sub>4</sub> [22–24] and PAF [25], and the major basic protein (MBP) rich in arginine, the molecular weight of which is 9300 in humans [26] and 11 000 in guinea pigs [27], is degranulated from the crystalloid core of specific eosinophil granules [28,29]. MBP causes injuries in the airway epithelium [30], schistosomules of *Schistosoma mansoni*, tumour cells in the abdominal cavity of mice, and *Trichinella spiralis* [31]. It was shown that inflammation of the airway epithelium by MBP causes its deceduation and the cessation of ciliary beating in guinea pigs [32]. It is also known that eosinophilic granule proteins such as eosinophilic cationic protein (ECP) and eosinophilic

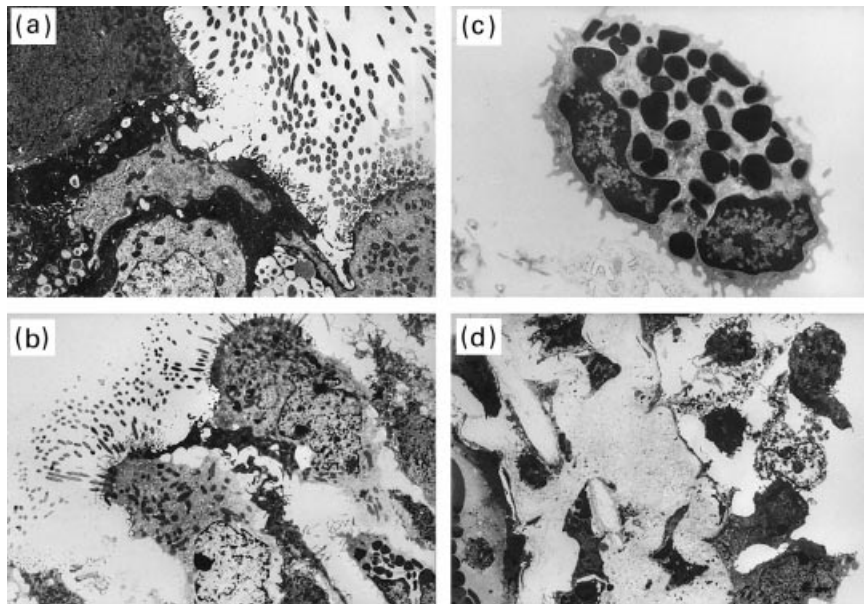


**Fig. 8.** (a) Control group (TEM): no abnormalities were noted in the control group. Junction was tightly connected. (b) IAR (TEM): rapid bronchial spasm and gaps occurred between ciliated epithelial cells, and eosinophil infiltration was present underneath the epithelium. (c) Eosinophils at IAR (TEM): degranulation was not observed. (d) LAR (TEM): further deciliation of ciliated epithelial cells, migration of numerous eosinophils from blood vessels, and exfoliation of basal cells were detected.

peroxidase (EPO) cause *in vitro* deciliation of the airway epithelium [33]. Erjefalt *et al.* demonstrated a significant correlation between the degree of epithelial damage and clusters of free eosinophilic granules [34].

In the present study, the relationship between asthmatic attacks and deciliation of the airway epithelium was examined. There were no differences between the non-sensitized and no-challenge groups in the conditions of cilia and the ciliated epithelium and infiltration of inflammatory cells (eosinophils, in particular) observed under light, scanning and transmission electron microscopy.

Although airway sensitivity to OA was probably

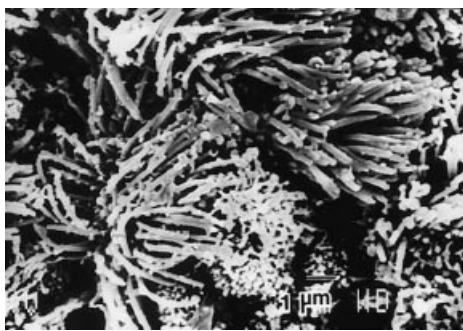


**Fig. 9.** Changes in the ciliated epithelial cells incubated with MBP. In the samples incubated in 100  $\mu\text{g/mL}$  MBP for 24 h, cilia that were broken off or had attached lumps of decomposed cilia fusion were observed (SEM).

enhanced at the completion of sensitization [35] morphological changes were not observed. In the animals in which sensitization had been completed, morphological changes of the airway epithelium were examined at the time of IAR, 5 min after OA inhalation (asthmatic attack). It was previously thought that activation of eosinophils occurs along with LAR after an inhaled antigen challenge, resulting in migration of granule proteins and deciliation of the airway epithelium [35–39]. However, some studies have reported the presence of a large amount of CrB at the time of IAR [2,12,14,35]. In the present study, involvement of eosinophils in deciliation of the airway epithelium at the time of IAR was examined.

In the BAL observation conducted by Tohda [13] using the same experimental dual-phase model of asthma as used in the present study, macrophages occupied almost the entire cell body in the sensitized no-challenge group, while a slight increase of eosinophils was noted in the IAR group. A light microscopic study identified a few sites of focal infiltration of eosinophils in the submucosa of the sensitized no-challenge group, and of diffuse mild infiltration in the regions from the submucota to the lamina propria mucosae of the IAR group. In the present study, slight infiltration of eosinophils was observed below the airway ciliated epithelium, but degranulation was not detected.

Greich *et al.* [30] demonstrated that  $10^{-7}$ – $10^{-5}$  M MBP from eosinophils destroys the airway epithelium in 3–23 h.

In a scanning electron microscopic study using airway rings of guinea pigs, Frigas *et al.* [33] found that MBP is cytotoxic to airway ciliated cells, and causes decay and decudation of cilia. This destruction of airway epithelial cells and cessation of ciliary beating were caused by MBP alone. It was also shown that 10 µg/mL MBP and 2 h needed 6 h for decudation of the airway epithelium by 100 µg/mL MBP. Thus, MBP alone displayed marked cytotoxicity to the airway epithelial cells during extended exposure. Therefore, decudation of the epithelium involving eosinophils is considered to occur at the time of IAR. An eosinophilic granule protein, eosinophilic cationic protein (ECP), also causes decudation of the airway epithelium [34]. There are positive correlations between the ECP concentration at the site of epithelial decudation, the CrB or the ECP concentration in the sputum of patients with bronchial asthma, and the decudation of the airway epithelium or the enhancement of bronchial sensitivity [10,11,40]. This suggests that eosinophilic granule proteins are also involved in the *in vivo* decudation of airway epithelium [41,42].

At the time of occurrence of IAR, although the eosinophils are not the main component involved in tracheal contraction and bronchial hypersensitivity, many eosinophils are already being recruited beneath the mucosa of the airway prior to the next allergic reaction.

In the present study, decudation of the ciliated epithelium and destruction of cilia were observed only 5 min after the occurrence of IAR. These changes are likely to be caused by mechanical destruction of airway smooth muscles, which induce bronchial spasms. Marked cytotoxicity of MBP to airway cells was also noted. It was suggested from this observation that repeated inflammation in the airway and decudation of the airway epithelium caused by granule proteins released by eosinophils after the occurrence of IAR leads to chronic asthma that resists medical treatment.

In conclusion, it is speculated that disruption and decudation of airway cilia are amplified by stimuli such as bronchial spasms induced by inflammatory mediators, and the following infiltration of inflammatory cells leads to chronic and intractable asthma.

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