

Effect of Disodium Cromoglycate on Airway Mucus Secretion during Antigen-Induced Late Asthmatic Responses in a Murine Model of Asthma

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Key Words

Asthma · Airway · Disodium cromoglycate · Mucin

Abstract

Background: Disodium cromoglycate (DSCG) is known to inhibit both immediate and late asthmatic responses (IAR and LAR). However, its effect on mucus hypersecretion is unknown. Using a murine model of asthma, we aimed to determine whether mucus secretion increased during IAR and LAR. We also studied the potency of DSCG in inhibiting mucus secretion and on airway eosinophilia. **Methods:** Mice were subjected to initial intraperitoneal sensitization and airway challenge to ovalbumin (OVA) and then provoked by additional exposure to OVA. Some mice were pretreated with aerosolized DSCG (20 mg/ml) 1 h before the provocation with OVA. After serial measurements of enhanced pause (Penh), an indicator of airflow obstruction, serum samples and bronchoalveolar lavage fluids (BALF) were collected. Then, the lungs were excised and a morphometric analysis for mucus hypersecretion was performed. **Results:** A biphasic increase in Penh (IAR and LAR) was observed in sensitized animals after provocation with OVA. Airway eosinophilia was observed during both responses. Intraluminal mucus significantly increased during LAR, but not during IAR. DSCG significantly attenuated both IAR and LAR, and significantly inhibited the increase in intra-

luminal mucus during LAR, but had no effect on eosinophilia in BALF. **Conclusion:** Our results suggest that airway hypersecretion may be involved as a component of airflow obstruction during LAR, and that this is unlikely during IAR. DSCG may be effective in reducing excessive airway mucus caused by exposure to allergens.

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Introduction

Mucus hypersecretion from mucus-secreting cells is an important component of airflow obstruction seen in airway diseases such as asthma. In a subgroup of asthma, allergen provocation leads to biphasic bronchoconstrictive responses, i.e. immediate asthmatic response (IAR), peaking at 15–30 min and returning to baseline within 1–2 h, and late asthmatic response (LAR), commencing 3–5 h after provocation and peaking at 6–12 h [1, 2]. The mechanisms and the airway cells involved in IAR and LAR may differ because the efficacy of each antiasthma drug differs against each of these responses. It is likely that IAR mainly depends on airway smooth muscle contraction in view of its rapid onset and prompt return to the baseline level in lung function. By contrast, mucus hypersecretion may, at least in part, contribute to the development of LAR considering its slow and persistent airway obstruction.

The mechanism of airway mucus secretion in respiratory diseases such as asthma has been studied mainly using morphometric analysis in humans and in experimental animals [3–5]. We showed that neurogenic inflammation induced airway goblet cell discharge in guinea pigs by developing a method in which the mucus score was measured as an index of secretion [3]. In humans, Aikawa et al. [6] quantified intraluminal mucus and demonstrated that mucus hypersecretion could be an important component of airway obstruction in chronic bronchitis. In guinea pigs, Hashimoto et al. [5] showed that pretreatment with diesel exhaust induced the formation of mucus plugs in the airway lumen 15 min after allergen challenge in sensitized animals. This finding indicates that mucus hypersecretion may contribute to the airway responses caused by allergen exposure in sensitized individuals.

Disodium cromoglycate (DSCG) is a widely used antiasthma drug, especially for younger children with asthma. DSCG has been shown to inhibit both IAR and LAR in humans [7] and in experimental animals [8]. The main mechanism of DSCG in inhibiting antiallergic action is thought to be via the mast cell stabilizing effect, although other airway effects, such as sensory nerve inactivation [9] and tachykinin antagonist activity [10], have been proposed.

In the present study, we wanted to clarify whether mucus secretion increased during airway obstruction caused by allergen exposure. For this purpose, we employed a murine model of asthma [8] in which the animals showed both IAR and LAR after provocation with antigen. We quantified intraluminal mucus, as well as airway eosinophilia, during IAR and LAR, and evaluated the inhibitory effects of DSCG against these responses.

Materials and Methods

Sensitization, Airway Challenge and Provocation with Ovalbumin (OVA)

All of the experimental animals in this study were used in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Principally, the method reported by Cieslewicz et al. [8] was employed for the sensitization and airway challenge of the animals. In brief, BALB/C mice, 10–12 weeks of age, were sensitized on days 1 and 14 by intraperitoneal injection of 20 µg OVA (grade V; Sigma, St. Louis, Mo., USA) emulsified in 2.25 mg aluminum hydroxide (Wako, Tokyo, Japan) in a total volume of 100 µl. On days 28, 29 and 30, the mice were challenged daily with OVA (1% in saline) for 20 min via the airways using an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan) in a 4.5-liter inhalation box. On day 32, the mice were provoked with OVA (5% in saline) or saline for 20 min. Following this, airway responsiveness was determined at 5, 15, 30 and

60 min, and then at intervals of 30 min for the next 6 h using whole-body barometric plethysmography. In another group of animals, airway responsiveness was measured for 12 h after the provocation to observe the time course of LAR.

Study Protocol

In a pilot study (study I) in which airway responsiveness was observed for 12 h after provocation, mice were sensitized, challenged with OVA and provoked with either OVA (n = 6) or saline (n = 5) to confirm that both IAR and LAR appeared in the present model.

In a second study (study II), the degree of mucus secretion and the number of eosinophils in BALF during IAR or LAR were measured and the inhibitory effects of DSCG on these responses were examined. All of the animals were sensitized, challenged with OVA and then treated with either DSCG (20 mg/ml diluted with saline) or the vehicle (saline) 1 h before provocation. After provocation with either OVA or the vehicle (saline), the animals were killed at 15 min or at 6 h following the measurement of airway responsiveness. Thus, there were six groups of animals: two groups pretreated with the vehicle for DSCG 1 h before provocation with OVA and killed either 15 min (IAR) or 6 h (LAR) after provocation (n = 10 and n = 13, respectively). Ten of the animals treated with DSCG before provocation with OVA were killed at 15 min and the remaining 16 animals 6 h after provocation. Control animals were treated with the vehicle for DSCG prior to provocation with saline and were then killed at the same time points (n = 10 and n = 10, respectively).

Determination of Airway Responsiveness

The time course of airway responsiveness after the antigen provocation was studied on unstrained, conscious mice as described previously [11, 12]. Mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Sharon, Conn., USA) and the pressure-time wave was continuously measured. The main indicator of airflow obstruction, enhanced pause (Penh), was calculated. It has recently been shown that Penh does not always correlate with airway resistance [13], but we used Penh as the parameter because this was suitable for the continuous measurement of the time course of airflow obstruction.

Bronchoalveolar Lavage (BAL) Procedure

After the measurement of airway responsiveness, the animals were killed with an overdose of pentobarbital (50 mg/animal i.p.) in order to obtain serum samples. After taking the samples, a 18-gauge cannula was introduced into the proximal portion of the trachea, and the lungs were lavaged with 0.4 ml of phosphate-buffered saline 3 times. The BAL fluids were centrifuged at 800 rpm for 5 min. Cell pellets were resuspended in 0.3 ml of RPMI-1640 medium. Total cell counts were performed with a hemocytometer, and differential cell counts were performed on cytopsin preparations stained with Diff-Quick (Kokusai-Shiyaku, Tokyo, Japan). A blinded observer counted a minimum of 200 cells for each sample.

Tissue Preparation

After the BAL procedure was completed, the lungs were inflated to a pressure of 25 cm H₂O. The trachea was clamped until fixation was complete. Tissue specimens were cut into 6-µm-thick sections at mid-sagittal slices in the mid-sagittal plane, embedded in paraffin and stained with Alcian blue and periodic acid-Schiff (AB/PAS). Slides were coded and graded in a blind fashion.

Quantification of Intraluminal Mucus

The mucosubstance area in the airway lumen was quantified by evaluating mucus areas coated in mucus morphometrically. In brief, the stained slides were examined with a light microscope (IX 70; Olympus, Tokyo, Japan) connected to a color chilled CCD camera system (M 3204C; Olympus) and to a color digital graphic tablet (Intuos GD-0608-R; Wacom, Saitama, Japan). Images of all membranous airways in the slide were recorded. Analysis was performed on a personal computer (PC 300 GL, IBM, New York, N.Y., USA) with images displayed on the color monitor using graphic analysis software (Win ROOF version 3.41; Miitani, Fukui, Japan). The length of the inner perimeter (Pi) of each airway lumen was measured and the luminal size of each airway calculated, assuming a circular airway [4, 6], in accordance with the formula: luminal size (mm^2) = $\text{Pi}^2/4\pi$.

All peripheral airways that were cut vertically in section profile with inner perimeters ranging from 0.79 to 3.5 mm were evaluated for morphometrical analysis of intraluminal mucus. Preliminary studies showed that there was no epithelial goblet cell metaplasia in unsensitized animals or in airways smaller than 0.79 mm even in sensitized animals. Therefore, airway lumens with $\text{Pi} < 0.79$ mm were not measured.

All areas of intraluminal mucus in each slide (areas stained purple by AB/PAS) were measured and added together. The mucus areas in each animal were then defined using the formula:

Mucus area (mm^2) = total area of intraluminal mucus/number of airway lumens examined.

OVA-Specific IgE Antibody Determination

OVA-specific IgE levels were determined by ELISA, as described previously [14]. In brief, 96-well microtiter plates (Dynex, Tokyo, Japan) were coated with 200 $\mu\text{g}/\text{ml}$ of OVA (grade V; Sigma) diluted in 0.1 M NaHCO_3 and incubated for 2 h at 37°C. After washing and blocking with PBS-bovine serum albumin for 2 h at 37°C, serially diluted 100- μl serum samples were added and incubated for 2 h at 37°C. After washing, 100 μl of 1:800-diluted rat anti-mouse IgE monoclonal antibody (Biosource International, Camarillo, Calif., USA) were added and incubated for 2 h at 37°C. Then, the reaction chromogen was generated with FAST (Sigma), and stopped with H_2SO_4 . Plates were read in a multiplate reader at 490 and 620 nm. Serum pooled from OVA-sensitized and airway-challenged adult mice was used as a positive control. The OVA-specific IgE titer was determined to be the reciprocal of the highest dilution giving a positive value (arbitrary unit: AU).

Statistical Analysis

All data were expressed as means $\pm$ SEM. Non-parametric analysis of variance (the Kruskal-Wallis test for unmatched pairs) was used to determine the significance of variance between groups. If a significant variance was found, a Mann-Whitney U test was performed to assess the significance of differences between groups. A p value of less than 0.05 was considered to indicate statistical significance, and a Bonferroni correction was performed when required. As for the level of significance of Penh during IAR and LAR, the area under the curve during corresponding time points was compared. Statistical analysis was performed utilizing Statview version 4.5 (Avacus Concepts, Berkeley, Calif., USA).

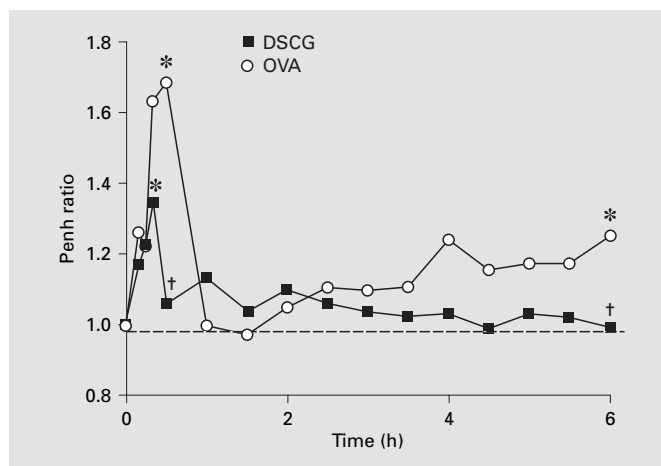


Fig. 1. The effect of DSCG on the increase in Penh up to 6 h after the provocation. Penh was expressed as the ratio compared to the baseline level before the provocation. The values are means at each time point. DSCG significantly attenuated the increase in Penh caused by OVA in both phases ($^\dagger p < 0.05$). $^* p < 0.05$ compared to control animals given vehicle for OVA.

Results

Airway Responsiveness after Allergen Exposure

In study I, provocation with 5% OVA induced both IAR and LAR. An increase in Penh was observed as early as 5 min after provocation, peaking at 15 min, returning to initial values by 90 min. The Penh at IAR was statistically significant compared to that in mice sensitized and challenged with OVA and provoked with saline ($p < 0.05$). In mice provoked with antigen, a second increase in Penh (LAR) started at around 2–3 h, and peaked at 8.25 h on average. Then, the value of Penh declined in all animals. In 3 of these animals, the value declined to the baseline level by 12 h after the provocation. However, it was still higher than the baseline level in another 3 animals at the time point. As in IAR, Penh at LAR was significantly greater than that in mice sensitized and challenged with OVA and provoked with saline ($p < 0.05$).

In study II, provocation with OVA induced IAR and LAR. The values in Penh at both IAR and LAR were significantly higher than those in animals treated with the vehicle for OVA ($p < 0.05$ at both IAR and LAR). DSCG significantly attenuated both IAR and LAR ($p < 0.05$ at both responses), whereas Penh during IAR (not during LAR) in DSCG-treated animals was significantly higher than that in control animals treated with the vehicle ($p < 0.05$; Fig. 1).

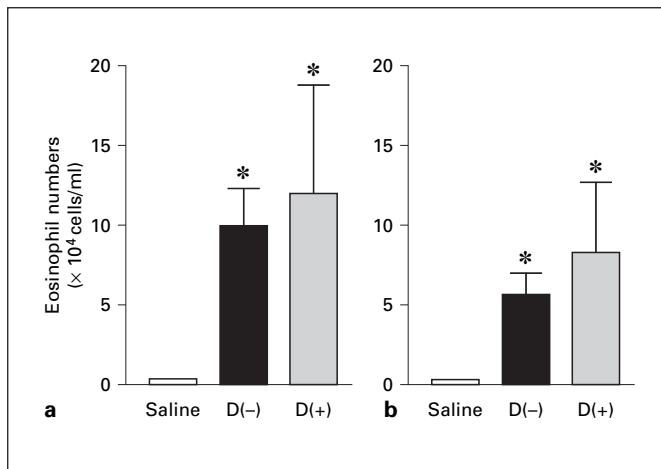


Fig. 2. The number of eosinophils in BALF during IAR (**a**) and LAR (**b**). Values are means $\pm$ SEM. D(-) and D(+) indicate OVA-challenged animals without or with pretreatment of DSCG, respectively. Saline = Control animals given vehicle for OVA and sacrificed at the corresponding time points. A marked elevation in eosinophil counts was observed both at IAR (**a**) and at LAR (**b**). DSCG did not affect the number of eosinophils both at IAR and LAR. * $p < 0.05$ compared to control animals given vehicle for OVA.

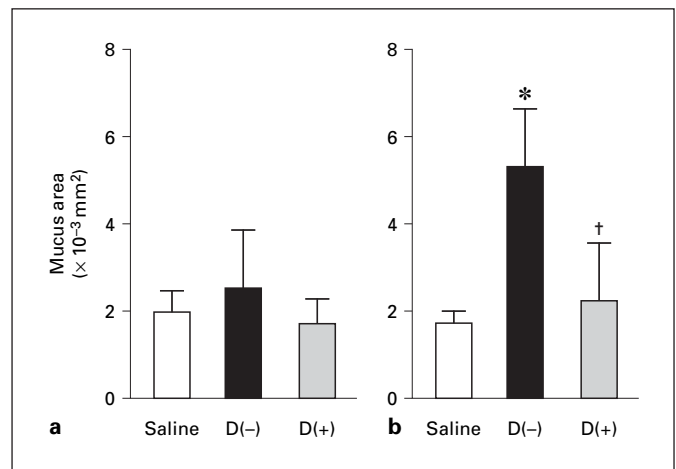


Fig. 3. The mucus area during IAR (**a**) and LAR (**b**). Values are means $\pm$ SEM. D(-) and D(+) indicate OVA-challenged animals without or with the pretreatment of DSCG, respectively. Saline = Control animals given vehicle for OVA and sacrificed at the corresponding time points. Mucus areas increased during LAR in OVA-challenged animals. DSCG significantly inhibited the increase during LAR. * $p < 0.05$ compared to control animals at the corresponding time points. † $p < 0.05$ compared to animals given OVA without pretreatment of DSCG.

Table 1. Mean value of the luminal size in each group

Group	n	Luminal size $\times 10^{-3}$ mm ^{2}
IAR		
Saline	10	108.3 ± 24.0
D(-)	10	135.0 ± 22.8
D(+)	10	112.9 ± 22.8
LAR		
Saline	10	102.4 ± 21.8
D(-)	13	124.5 ± 21.0
D(+)	16	102.5 ± 5.6

Values are means $\pm$ SEM. D(-) and D(+) indicate OVA-challenged animals without or with pretreatment of DSCG, respectively. Saline means control animals given vehicle for OVA and sacrificed at the corresponding time points. No significant difference in the size of airway lumina was observed among the six groups.

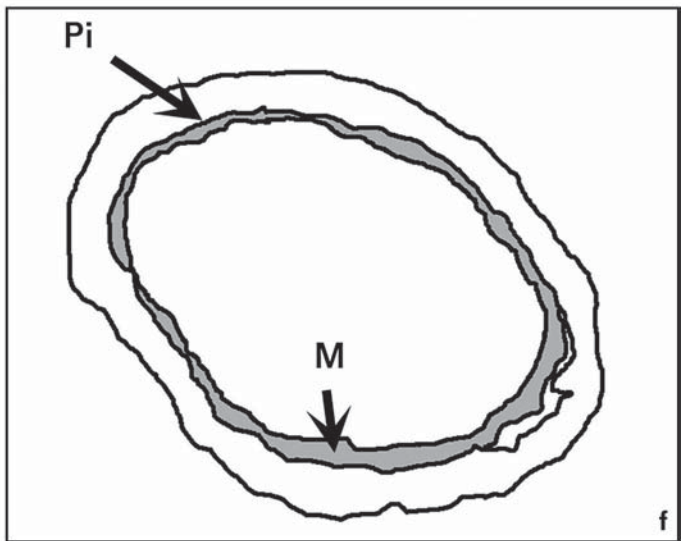
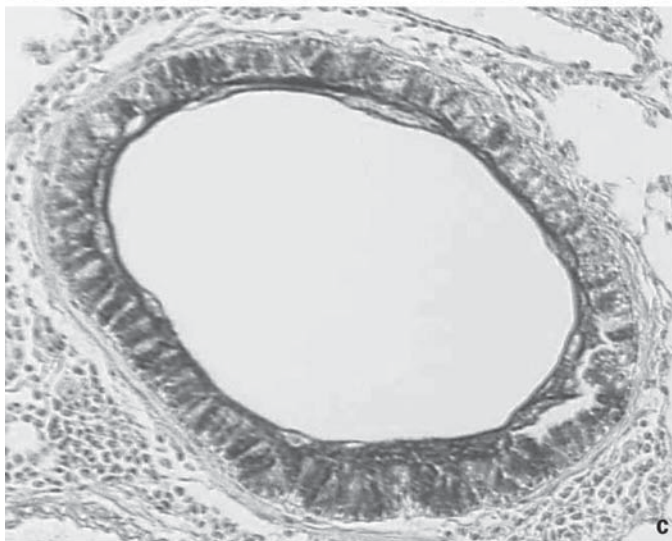
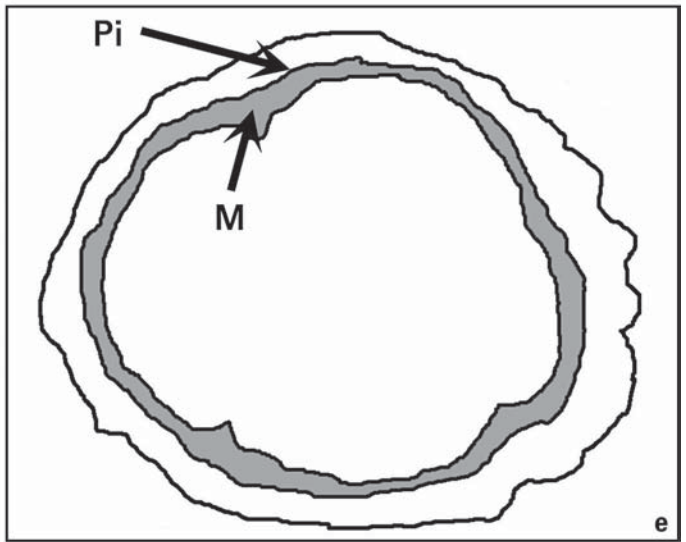
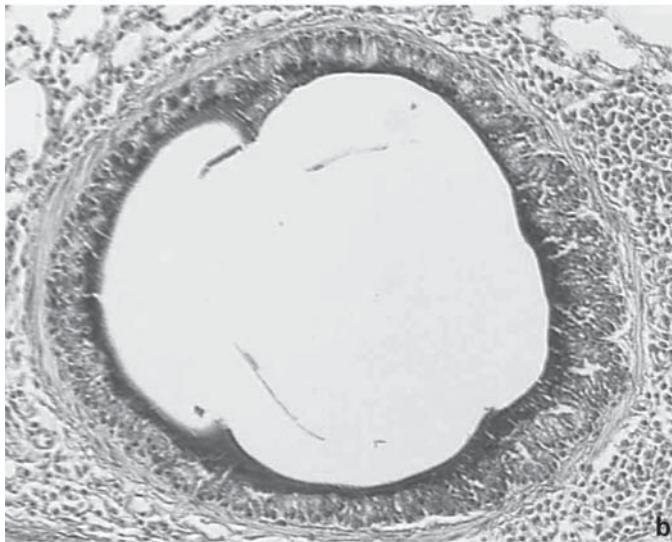
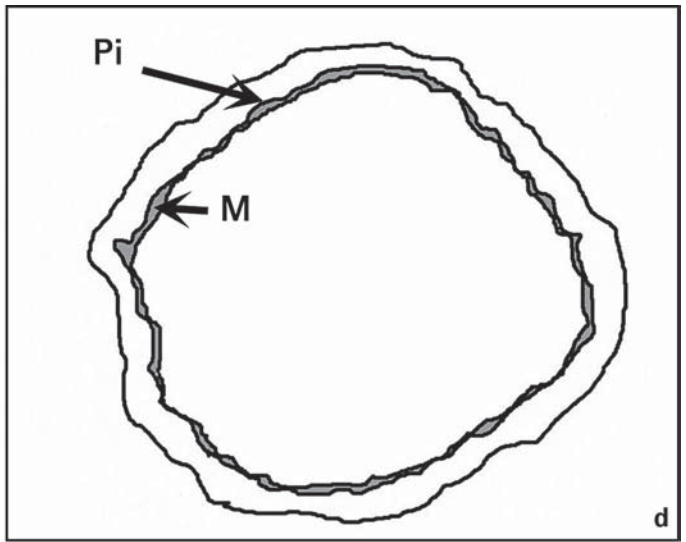
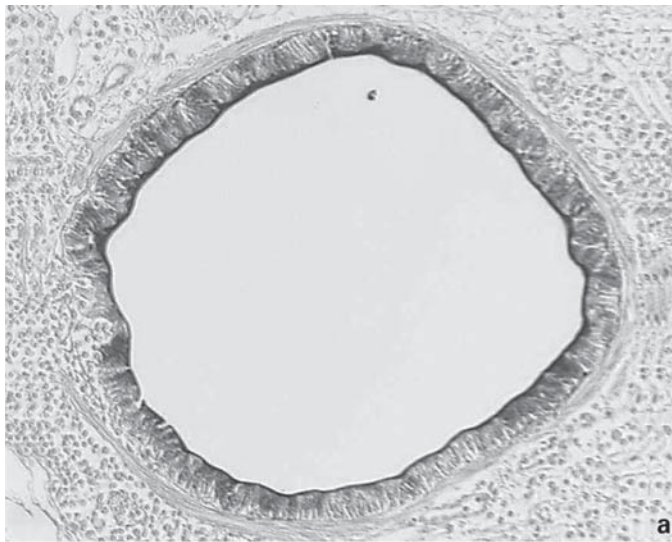
Airway Eosinophilia

In animals not pretreated with DSCG, a marked elevation in the number of eosinophils in the BALF was observed during both IAR and LAR ($9.87 \pm 2.71 \times 10^4$ and $5.59 \pm 1.28 \times 10^4$ cells/ml, respectively). These

numbers were significantly higher than those in control animals killed at corresponding time points ($0.018 \pm 0.011 \times 10^4$ and $0.015 \pm 0.015 \times 10^4$ cells/ml, respectively; $p < 0.05$ and $p < 0.05$, respectively). DSCG had no effect on the number of eosinophils during IAR or LAR (fig. 2).

Intraluminal Mucus

The luminal size was not significantly different among the six groups (table 1). Morphometric analysis revealed that the mucus area in the airway lumen ($\times 10^{-3}$ mm ^{2}) was significantly greater at LAR (5.28 ± 1.07 , $p < 0.05$) but not at IAR (2.49 ± 1.21) in animals provoked with OVA than in those given the vehicle for OVA (1.93 ± 0.58 and 1.70 ± 0.51 at the time points of IAR and LAR, respectively). In DSCG-treated animals, the mucus areas at the time points of IAR and LAR were comparable with those in control animals (1.66 ± 0.74 and 2.21 ± 1.27 , respectively). DSCG significantly inhibited the increase in the mucus areas at LAR ($p < 0.05$, fig. 3). A photograph typical of each group is shown in figure 4. Areas quantified for Pi measurement are marked in the figure.



4

Analysis of OVA-Specific IgE

The serum level of OVA-specific IgE was markedly elevated in all groups of animals subjected to sensitization and challenge (range: 80–20,480 AU). In the three groups of animals examined at the time point of IAR, the mean values of the IgE titer (AU) were $4,608 \pm 512$, $10,752 \pm 3,999$ and $15,360 \pm 3,238$ in animals without provocation, and those with provocation of OVA without

and with pretreatment of DSCG, respectively. In the three groups of animals examined at the time point of LAR, the mean values of the IgE titer (AU) were $12,288 \pm 2,048$, $8,192 \pm 1,254$ and $8,288 \pm 4,977$ in animals without provocation, and those with provocation of OVA without and with pretreatment of DSCG, respectively. No significant difference in the IgE titer was found among the six groups.

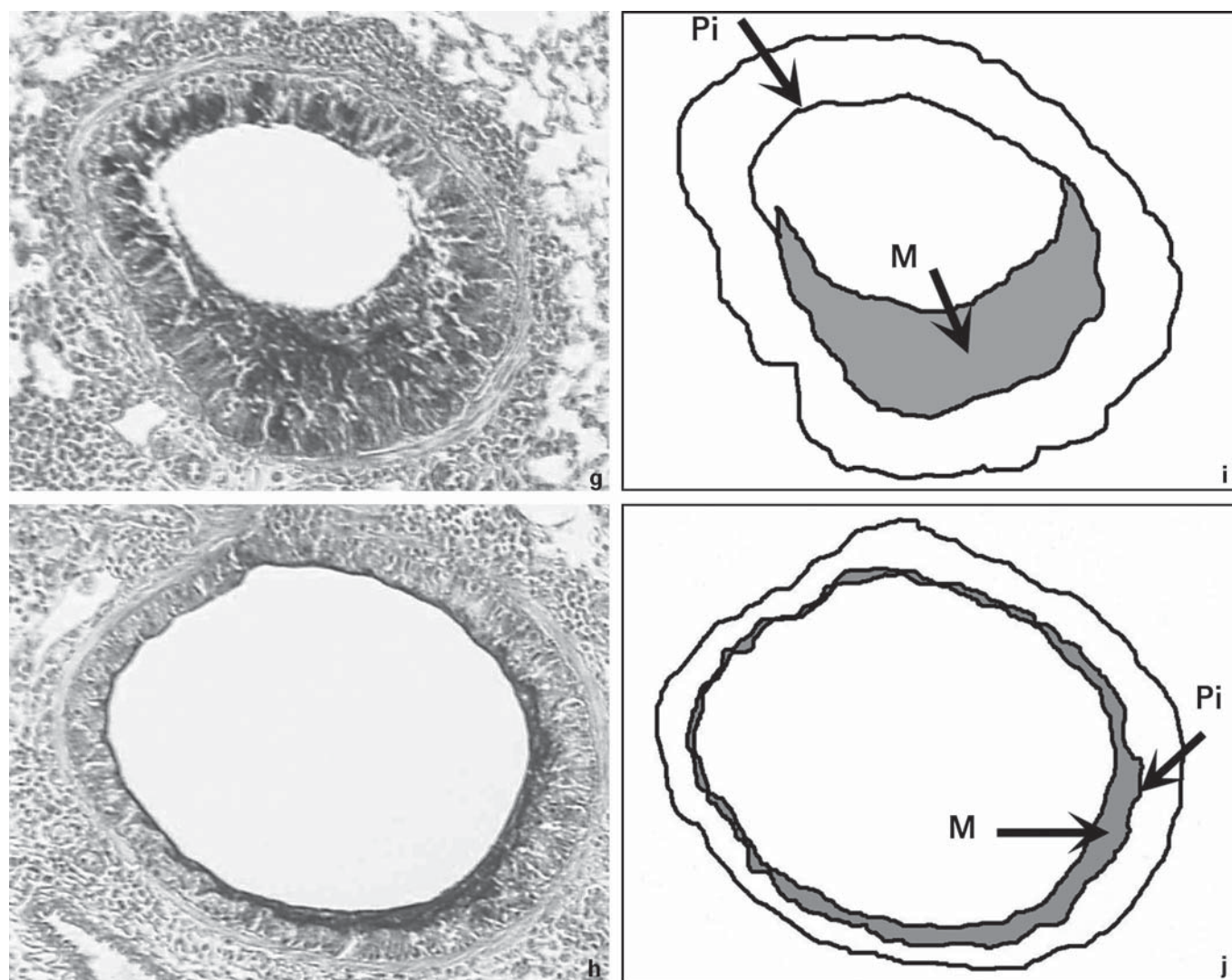


Fig. 4. Representative photomicrographs of 6-μm sections of mice lung (AB/PAS staining). The sections of the control animal sacrificed 15 min after the provocation with vehicle for OVA (**a**), those at IAR without or with DSCG (**b**, **c**, respectively), and those at LAR without or with DSCG (**g**, **h**, respectively) are shown. In mice during LAR (**g**), remarkably greater amounts of mucus are observed. **d–f**, **i**, **j** Pi and mucus area (M) of the corresponding panels were marked to show how to measure these parameters.

Discussion

In a murine model of asthma in which both IAR and LAR were observed after antigen provocation, we demonstrated that the quantity of mucus in the airway lumen significantly increased during LAR, but not during IAR. DSCG significantly attenuated the amount of intraluminal mucus in LAR, but did not affect airway eosinophilia during IAR or LAR. It is difficult to directly evaluate the amount of degranulated intraluminal mucus. Thus, we performed a morphometric analysis to quantify the amount of mucus during antigen-induced airway responses. In the present study, we quantified the intraluminal mucus after performing the BAL procedure. It has been shown that the recovery rates of substances in intraluminal fluids differ depending upon several conditions, e.g. viscosity [15]. Thus, it is likely that mucus components that are difficult to remove from the airways by BAL may be responsible for the difference in the amount of intraluminal mucus in the present study. Measuring the content of mucins in the BALF would be useful to determine the degree of secretion, but we did not do so in the present study.

In humans, even mild asthma is associated with goblet cell hyperplasia and increased stored mucin in the airway epithelium, whereas moderate asthma is associated with increased stored mucin and secreted mucin [16]. These findings suggest that acute degranulation from hyperplastic goblet cells may be the cause of asthma exacerbations in mild and moderate asthma, and that chronic degranulation from goblet cells may contribute to chronic airway narrowing in moderate asthma. In rodents, allergic sensitization with OVA has been shown to cause goblet cell metaplasia [17], and the Th2 cytokines IL-4 and IL-13 have been implicated in this process [18]. In the present study, we demonstrated that mucus-secreting cells in sensitized mice degranulated greater amounts of intraluminal mucus during LAR, but not during IAR, indicating that airway mucus hypersecretion may be a component of airflow obstruction during LAR, but not during IAR. However, we do not know the exact role of the excess mucus during LAR. It has been shown that other events such as increased plasma exudation and eosinophilic inflammation occur during LAR. Thus, a combination of these events with mucus hypersecretion may be responsible for long-lasting airflow obstruction, a characteristic feature of LAR. In contrast to our results, several studies in asthmatic subjects have shown that there might be no degranulation of goblet cells after allergen challenge [19]. We can only surmise that this difference between results

may be due to a difference in species: further research is required to clarify this matter.

In the present study, we could not clarify the mechanism of mucus hypersecretion during LAR. Various kinds of secretagogues causing the degranulation of airway mucus have been proposed and include environmental stimuli such as smoke and ammonia, local release of neuropeptides or activation of cholinergic reflex pathways, and inflammatory mediators such as neutrophil elastase, mast cell chymase, leukotrienes, histamine and non-protease neutrophil products [20]. Thus, one or more factors among them may contribute to the degranulation after antigen exposure. In the present study, DSCG had no effect on the number of eosinophils in BALF, during IAR or during LAR, confirming previous reports in guinea pigs [21] and in mice [22], whereas it significantly decreased the amounts of intraluminal mucus during LAR. Our results may indicate that the activation of eosinophils is not required for the degranulation process of airway mucus after antigen exposure. In experimental animals, it has been shown that eosinophils are not required for allergen-induced goblet cell metaplasia. Cohn et al. [23] showed that airway mucus production did not decrease even when airway eosinophils are progressively eliminated from the lung. Based on this finding, they speculated that the association between airway eosinophilia and increased mucus production seen in mice merely reflects the association between eosinophils and activated Th2 cells secreting IL-5. Further studies are required to clarify the role of eosinophils in mucus degranulation.

A single application of DSCG is shown to be sufficient to inhibit both IAR and LAR, not only in humans [7] but also in experimental animals [8]. Our present study confirms previous observations. Also, we showed that DSCG may be potent in inhibiting intraluminal degranulation of airway mucus during LAR. As far as we know, there has been no report that DSCG has any effect on mucus production or mucus gene expression. We were not able to clarify the exact mechanism by which DSCG inhibited mucus hypersecretion. Because DSCG is known to have a mast cell stabilizing effect, it is likely that it inhibits mucus secretion secondary to inhibiting mast cell function. Mast cells are known to induce allergic airway inflammation via the release of various cytokines and chemokines such as IL-4, IL-13 and eotaxin; inflammatory mediators such as histamine, cysteinyl leukotrienes and platelet-activating factor; and free radicals such as the superoxide anion. It has been suggested that several of these substances may be the secretagogues causing degranulation of airway mucus. Thus, it is reasonable to

assume that one or several factors generated from mast cells may trigger mucus hypersecretion during LAR. Alternatively, it is possible that DSCG has a direct stabilizing effect against airway epithelial cells, which are responsible for airway mucus secretion, and consequently attenuates the degranulation of these cells. Further studies are required to examine this possibility.

In conclusion, we have shown that airway mucus hypersecretion may be a component of airflow obstruction during LAR, but not during IAR, in a murine model of

asthma. DSCG may be effective in reducing excessive airway mucus during asthmatic attacks in humans: further studies are required to examine this possibility.

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