

# Absence of Nasal Blockage in a Japanese Cedar Pollen-Induced Allergic Rhinitis Model Mouse

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## ABSTRACT

**Background:** Japanese cedar pollen-induced allergic rhinitis in a guinea pig model clearly induced not only sneezing but also biphasic nasal blockage. To date, there have only been a few reports on models of murine allergic rhinitis which clearly show nasal blockage. Therefore, in order to try and develop such a model, we administered multiple dosages of intranasal pollen or purified antigen protein Cry j 1.

**Methods:** B10.S mice were sensitized by intranasal instillations of either pollen extract or Cry j 1 twice a day for 7 days, which was adsorbed on Al(OH)<sub>3</sub>. Subsequently, once a week, the mice were given multiple intranasal instillation challenges of either the pollen suspension or Cry j 1 and the frequency of sneezing was observed after respective challenges were made. Specific airway resistance (sRaw) was measured as an indicator for nasal blockage. Cry j 1-specific IgE levels were measured using an enzyme-linked immunosorbent assay.

**Results:** The serum Cry j 1-specific IgE level showed clear elevation only in the group sensitized by Cry j 1 + Al(OH)<sub>3</sub> and then challenged by Cry j 1. No elevations were seen in the groups sensitized by pollen extract + Al(OH)<sub>3</sub> followed by a pollen suspension challenge. There was an immediate increase in sneezing after challenges in all of the sensitized-challenged groups. Nevertheless, no increases in sRaw in any of the groups were detected at any of the time points during the 8 hours following the challenges.

**Conclusions:** Cry j 1 may be more effective than crude antigens for efficient sensitization/challenge in mice. No increase in sRaw occurred, even in mice that possessed high amounts of Cry j 1-specific IgE and that exhibited sneezing.

## KEY WORDS

allergic rhinitis, animal model, cedar pollen, nasal blockage, sneezing

## INTRODUCTION

We established a guinea pig model of Japanese cedar pollen-induced allergic rhinitis.<sup>1-3</sup> In this model, sneezing was immediately induced, and nasal blockage, the most serious symptom for allergic rhinitis patients, was biphasically caused after a pollen inhalation challenge.<sup>2</sup> We analyzed the induction mechanisms underlying the allergic nasal symptoms.<sup>4-6</sup> Similar to our model, other groups have also reported that sensitized guinea pigs show not only sneezing but also biphasic nasal blockage after challenges with the allergen ovalbumin (OVA).<sup>7-9</sup>

Mice have provided excellent models for analyzing

the mechanisms of inflammatory diseases, as many immunological tools and gene-modified animals are available for testing this system. In murine allergic rhinitis models, incidence of sneezing and nasal rubbing, increased sneezing as a response to histamine, infiltration of eosinophils into the nasal mucosa and Th2 cytokine production, have been observed as allergic symptom parameters in the upper airway.<sup>10-14</sup> It has remained unclear until recently, whether nasal blockage was induced after an intranasal challenge with an antigen in mice. However, Miyahara<sup>15,16</sup> has reported that intranasal OVA challenge caused an early phase nasal blockage that peaked at 4–10 minutes after the challenge, along with an increase in na-

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sal airway resistance that was present even at 24 hours in anesthetized mice.

Japanese cedar pollen, which is one of the most common allergens that induces pollenosis in Japan, contains 2 major antigenic proteins, Cry j 1<sup>17</sup> and Cry j 2.<sup>18</sup> It has been reported that in each of the antigenic proteins, there are 10 different T-cell epitopes that are active in patients with Japanese cedar pollenosis.<sup>19</sup> A major T-cell epitope has been identified in Cry j 1-sensitized B10.S mice,<sup>20</sup> and this epitope sequence coincides with the most prevalent epitope in patients. Therefore, it was suggested that the B10.S mouse would be a suitable strain for Japanese cedar pollen-associated diseases. Using B10.S mice sensitized by i.p. injections with Cry j 1 + Al(OH)<sub>3</sub>, Tsunematsu *et al.*<sup>21,22</sup> were able to create a murine model of pollen-induced allergic rhinitis, in which the animals exhibited sneezing, eosinophil recruitment in nasal tissue and the production of serum Cry j 1-specific IgE after multiple intranasal challenges with Cry j 1. However, to date there have been no reports on whether nasal blockage can also be induced in a murine Japanese cedar pollen-induced allergic rhinitis model.

In the present study, we used B10.S mice to assess whether nasal blockage is caused in an allergic rhinitis model induced by pollen. In order to mimic as closely as possible the sensitization/challenge situations that can be seen in a clinical setting, mice were sensitized by multiple intranasal instillations of either a pollen extract + Al(OH)<sub>3</sub> or Cry j 1 + Al(OH)<sub>3</sub>, followed by repetitive challenges to animals with intranasal applications of either the pollen suspension or Cry j 1. In addition to monitoring the incidence of sneezing and the serum Cry j 1-specific IgE production, we also examined the increases in specific airway resistance (sRaw), which is a parameter of nasal blockage. Since sRaw has been used as a reliable indicator for nasal blockage in guinea pig models of allergic rhinitis,<sup>2-6</sup> this makes it possible to longitudinally measure the changes in airway resistance without having to employ any anesthesia or perform any surgical operations.

## METHODS

### ANIMALS

Five-week-old female B10.S mice were purchased from Japan SLC (Hamamatsu, Japan). The animals were housed in an air-conditioned room at a temperature of 23 ± 1°C and 60 ± 10% humidity with the lights turned on from 8:00 a.m. to 8:00 p.m. Animals were given a standard laboratory diet and water *ad libitum*. The first sensitization procedure was initiated 1 week after the arrival of the mice.

This animal study was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

### ANTIGEN AND ADJUVANT

Japanese cedar (*Cryptomeria japonica*) pollen was harvested in Gifu and Shiga prefectures (Japan) in 1998. Purified Cry j 1 was provided by Meiji Dairies Co. (Odawara, Japan). Al(OH)<sub>3</sub> gels were prepared with 0.25 N NaOH and 0.25 N Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>, as our group previously described.<sup>23</sup>

The cedar pollen extract used for the sensitization was prepared as follows. The pollen was suspended in PBS followed by vigorous stirring for more than 30 minutes. The suspension was then centrifuged, and the supernatant was collected. Protein concentration in the supernatant was measured using the method of Bensadoun and Weinstein.<sup>24</sup>

### SENSITIZATION AND CHALLENGE

#### Experiment 1 (Fig. 1)

The pollen-induced allergic rhinitis model in guinea pigs<sup>2-6</sup> served as the basis for determining the dosage of pollen extract and Al(OH)<sub>3</sub> that were used for sensitization in the mice. Based on body weight, the doses were decreased to 1/15 of the dosage used in the guinea pig model. Twice a day for 7 days, the mice were sensitized by bilateral intranasal instillation of the cedar pollen extract adsorbed onto Al(OH)<sub>3</sub> gel at a concentration of 0.02 µg protein/0.02 mg Al(OH)<sub>3</sub>/2 µl/each nostril. Prior to each sensitization, the upper airway mucosal surface was anesthetized by inhaling a mist of 4% lidocaine hydrochloride solution (Fujisawa Pharm. Co., Osaka, Japan) for 3 minutes, which was generated by an ultrasonic nebulizer (NE-U12, Omron, Osaka, Japan). The airway was anesthetized in order to prevent the rapid elimination of the antigen by ciliary movement.

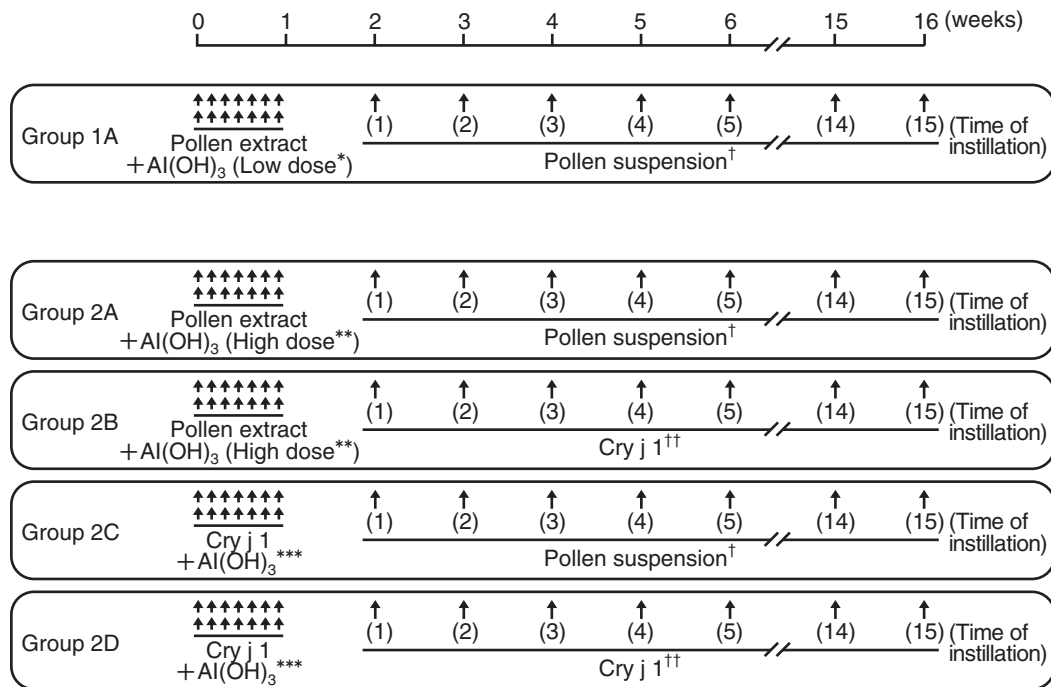
Two weeks after the first sensitization, the sensitized animals were then intranasally challenged with a pollen suspension at a dosage of 90 µg/2 µl/nostril (180 µg/animal) once every week until the 15th challenge (Group 1A).

#### Experiment 2 (Fig. 1)

In the second group, mice were intranasally sensitized with a 50-fold higher dosage of the extract + Al(OH)<sub>3</sub> which was used in Group 1A (1 µg protein/0.1 mg Al(OH)<sub>3</sub>/2 µl/each nostril/time, Groups 2A and 2B). In addition, in place of the pollen extract, purified Cry j 1 + Al(OH)<sub>3</sub> was used for sensitization at 1 µg Cry j 1/0.1 mg Al(OH)<sub>3</sub>/2 µl/each nostril/time (Groups 2C and 2D). We have previously reported that approximately 80% of protein in the extract is Cry j 1.<sup>25</sup> Sensitizations were carried out by following the same schedule that was used in Experiment 1.

Two weeks after the first sensitization, the mice were then intranasally challenged with the pollen suspension at 90 µg/2 µl/nostril (180 µg/mouse) once every week until the 15th challenge (Groups 2A and 2C). Alternatively, Cry j 1 was used as a challenging antigen at a dosage of 2 µg/2 µl/nostril (4 µg/mouse)

## Pollen-Induced Murine Allergic Rhinitis



**Fig. 1** Schedule for intranasal sensitization, using either Japanese cedar pollen extract or Cry j 1, and the subsequent nasal challenges with intranasal administrations of either the pollen suspension or Cry j 1 in mice. \*: 0.02  $\mu\text{g}$  protein/0.02 mg  $\text{Al}(\text{OH})_3$ /2  $\mu\text{l}$ /nostril/time, \*\*: 1  $\mu\text{g}$  protein/0.1 mg  $\text{Al}(\text{OH})_3$ /2  $\mu\text{l}$ /nostril/time, \*\*\*: 1  $\mu\text{g}$  Cry j 1/0.1 mg  $\text{Al}(\text{OH})_3$ /2  $\mu\text{l}$ /nostril/time, †: 90  $\mu\text{g}$ /2  $\mu\text{l}$ /nostril, ††: 2  $\mu\text{g}$ /2  $\mu\text{l}$ /nostril.

(Groups 2B and 2D). From our previous report<sup>25</sup> we were able to calculate that the amount of Cry j 1 in 90  $\mu\text{g}$  of pollen was approximately 55 ng, and therefore the dosage (2  $\mu\text{g}$ /nostril) for Cry j 1 used in Groups 2B and 2D was higher than the amount contained in the 90  $\mu\text{g}$ -weight pollen suspension used in Groups 2A and 2C.

For the negative control groups, non-sensitized mice were intranasally treated with PBS (Group NS-NC) or the pollen extract with a dosage of 90  $\mu\text{g}$ /2  $\mu\text{l}$ /nostril (Group NS-C) according to the schedule described above.

### MEASURING SNEEZING FREQUENCY

The frequency of sneezing was measured during the first hour after pollen challenges were made.

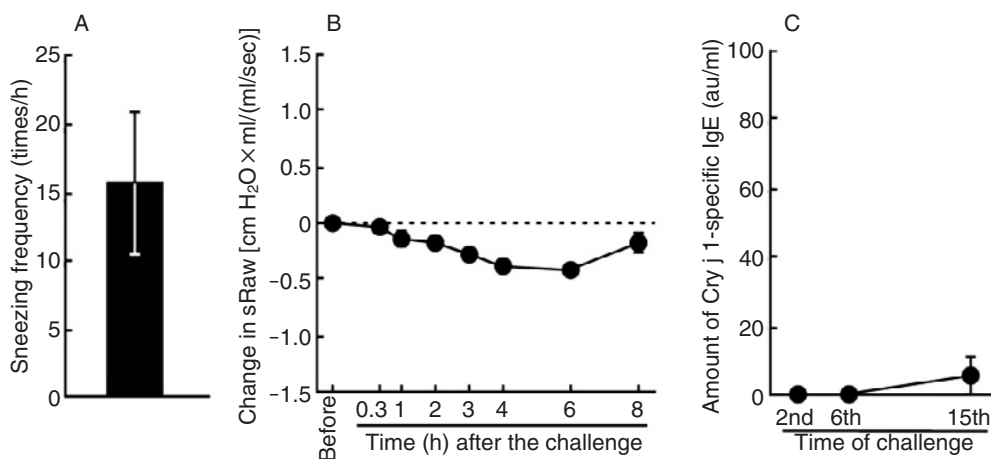
### MEASUREMENT OF SPECIFIC AIRWAY RESISTANCE (sRaw)

sRaw was used as an indicator for nasal blockage. A two-chambered, double-flow plethysmograph system along with a data analyzer Pulmos-I (MIPS, Osaka, Japan) were used to measure the sRaw as by the method of Pennock *et al.*<sup>26</sup>

### MEASUREMENT OF Cry j 1-SPECIFIC IgE ANTIBODY IN SERA

Cry j 1-specific IgE antibody was determined by an

enzyme-linked immunosorbent assay (ELISA) using blood samples collected 1 day before the challenges. In brief, the wells of an ELISA plate (Immulon 4HBX, Thermo Labsystems, Milford, MA, USA) were coated with 2  $\mu\text{g}/\text{ml}$  of Cry j 1 by incubation at 4°C for 12–18 hours. After the wells were washed, they were treated with a blocking buffer of 10% fetal bovine serum-containing PBS, for 2 hours at room temperature. Subsequently, diluted sample sera were added and then allowed to stand at 4°C. The following day, the plate was washed, and biotin-conjugated rat anti-mouse IgE (BD Pharmingen, San Diego, CA, USA) was added at 2  $\mu\text{g}/\text{ml}$ . Avidin-horseradish peroxidase conjugate (1 : 1000, BD Pharmingen) was added 1 hour after being washed, and the plate was then allowed to stand at room temperature for 30 minutes. After washing, the enzyme reaction was initiated by addition of a substrate, 3,3',5,5'-tetramethylbenzidine (Sigma Chem., St. Louis, MO, USA). The reaction was terminated by adding a sulfuric acid solution 30 minutes after the onset of the reaction. A microplate reader was used to measure absorbance at 450 nm, with the amount of Cry j 1-specific IgE (arbitrary unit (au)/ml) then calculated from a standard curve. The standard curve values were obtained from pooled anti-Cry j 1 mouse serum that had been prepared in advance by sensitizing B10.S mice with multiple intraperitoneal injections of the pollen extract +  $\text{Al}(\text{OH})_3$



**Fig. 2** (A) Induction of sneezing, (B) time-course changes in specific airway resistance (sRaw) after the 15th instillation challenge with Japanese cedar pollen suspension in sensitized mice (Group 1A) and (C) time-course changes in amount of Cry j 1-specific IgE during challenges with the pollen suspension (Group 1A). Each column or point represents the mean  $\pm$  S.E. of 5–10 animals.

at a dosage of 20  $\mu$ g protein/20 mg Al(OH)<sub>3</sub>/250  $\mu$ l/animal/time once a week for a total of 8 times.

### STATISTICAL ANALYSES

Statistical analysis was performed by using a one-way analysis of variance. If significant differences were detected, individual group differences were determined using Bonferroni's multiple test. A probability value (*P*) of less than 0.05 was considered to indicate a statistically significant difference.

### RESULTS

#### SNEEZING, NASAL BLOCKAGE AND IgE PRODUCTION IN LOW DOSAGE POLLEN EXTRACT-SENSITIZED MICE (EXPERIMENT 1)

Mice were intranasally sensitized with the pollen extract + Al(OH)<sub>3</sub>, followed by challenges with the pollen suspension once a week until the 15th challenge (Group 1A). The experimental protocol and schedule were based on the results obtained from our previous studies conducted on the guinea pig model of allergic rhinitis.<sup>2-6</sup> After the 15th challenge, there was an increase in induced sneezing of approximately 15 times/hour, although no increase in sRaw was indicated. Cry j 1-specific IgE slightly increased 1 day before the 15th challenge (Fig. 2).

#### SNEEZING, NASAL BLOCKAGE AND IgE PRODUCTION IN EITHER HIGH DOSAGE POLLEN EXTRACT- OR CRY J 1-SENSITIZED MICE (EXPERIMENT 2)

Based on the results of Experiment 1, the dosage of the pollen extract for sensitization was increased (Groups 2A and 2B). In addition, Cry j 1 was used as the sensitization (Groups 2C and 2D) and the challenging (Groups 2B and 2D) antigen. As shown in

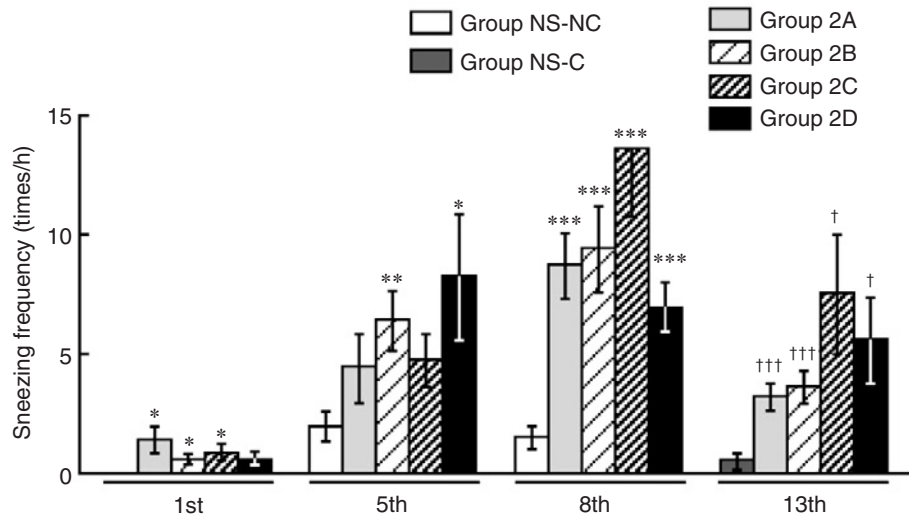
Figure 3, mice sensitized with either the extract + Al(OH)<sub>3</sub> or Cry j 1 + Al(OH)<sub>3</sub> showed increased frequencies of sneezing within 1 hour after the 1st challenge when exposed to either the pollen suspension (Groups 2A and 2C) or Cry j 1 (Groups 2B and 2D). The frequency of sneezing increased even more at the 5th, 8th, and 13th challenges. When non-sensitized mice were intranasally instilled with the pollen extract at the time corresponding to the 13th challenge, sneezing was induced by <1 time/hour.

Figure 4 represents the time-course changes in sRaw after the 1st, 5th and 15th challenges in Groups NS-NC, 2A, 2B, 2C, and 2D. On the 1st and 5th challenges, no increase in sRaw was detected after the challenges in any of the groups at any time point during the 0–8 hour time period. At the 15th challenge, elevations of sRaw were noted in all groups, including the NS-NC Group, at 20 minutes after the challenge.

Figure 5 shows the time-course changes occurring in the amount of Cry j 1-specific IgE antibody in the sera during the repetitive antigen challenges. High amounts of Cry j 1-specific IgE antibody were not detected in the sera collected from Groups 2A, 2B and 2C. A distinct contrast was seen after sensitization and challenge with Cry j 1 in Group 2D, in which the Cry j 1-specific IgE level markedly increased until the 14th challenge.

### DISCUSSION

We set out to determine whether nasal blockage is induced in the murine Japanese cedar pollen-induced allergic rhinitis model. In order to do this, B10.S mice were sensitized by multiple intranasal instillations with either a pollen extract + Al(OH)<sub>3</sub> or Cry j 1 + Al(OH)<sub>3</sub>, and then repetitively challenged by intranasal application with either the pollen suspension or



**Fig. 3** Induction of sneezing after the 1st, 5th, 8th and 13th challenges with either Japanese cedar pollen suspension (Groups 2A and 2C) or Cry j 1 (Groups 2B and 2D) in mice sensitized with either the pollen extract + Al(OH)<sub>3</sub> (Groups 2A and 2B) or Cry j 1 + Al(OH)<sub>3</sub> (Groups 2C and 2D). Each column represents the mean  $\pm$  S.E. of 7–14 animals. \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs. Group NS-NC, †  $p < 0.05$  and †††  $p < 0.001$  vs. Group NS-C.

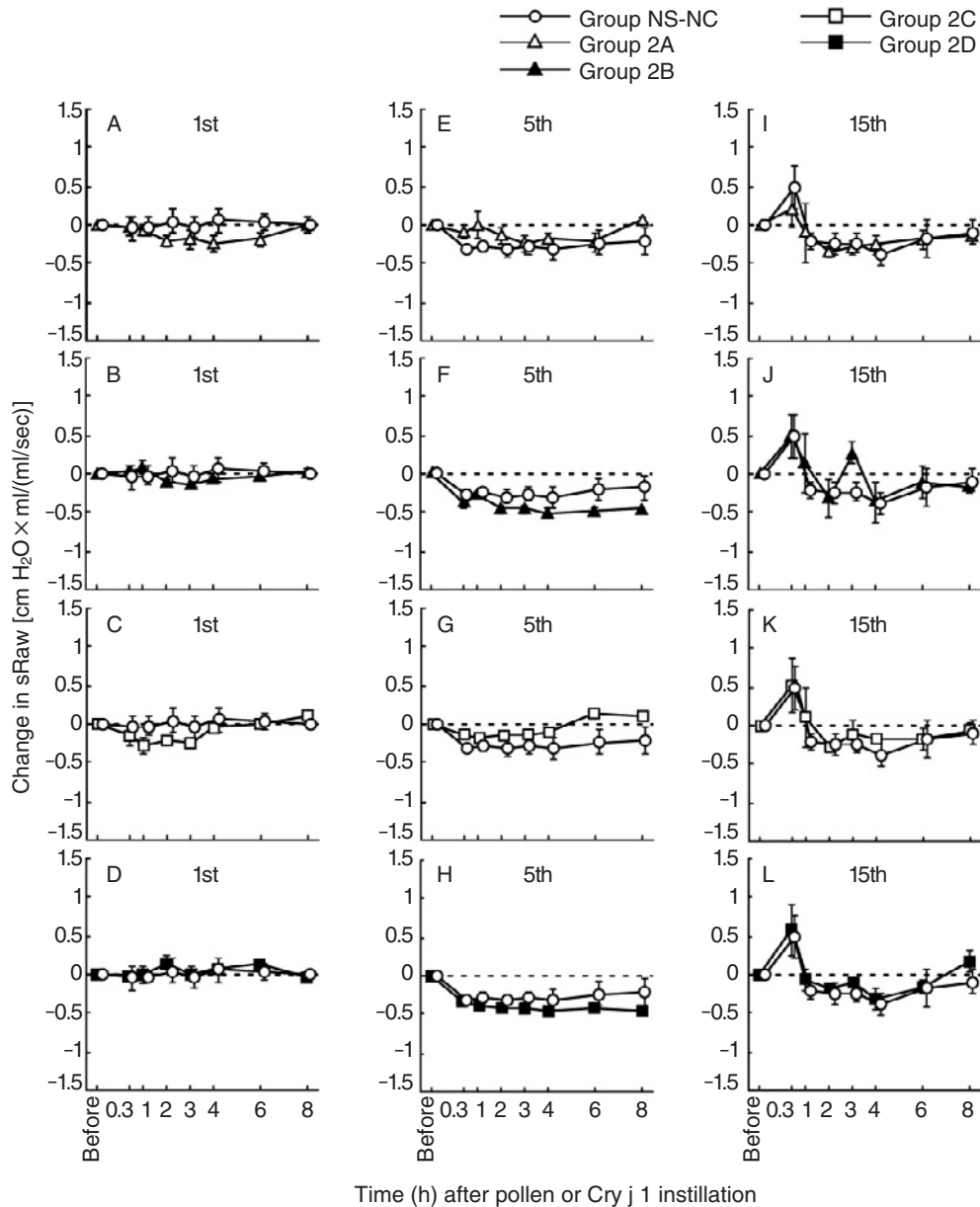
Cry j 1. The serum Cry j 1-specific IgE level was clearly elevated only in the group sensitized by Cry j 1 + Al(OH)<sub>3</sub> and then challenged by Cry j 1. No changes were found for groups which were sensitized by pollen extract + Al(OH)<sub>3</sub> and then challenged by the pollen suspension. However, the sneezing frequency immediately increased after the challenges in all of the sensitized-challenged groups. Nevertheless, there were no antigen-induced increases in the sRaw detected in any of the groups at any time point during the 8 hour period following the challenges. Although all groups including the negative control group showed an increase in sRaw 20 minutes after the 15th challenge, it is not clear why the antigen-independent response was induced. These results suggest that Cry j 1 may be more effective than crude antigens when trying to efficiently sensitize/challenge mice. However, even in mice that possessed both high amounts of Cry j 1-specific IgE in the sera and exhibited sneezing, there were no increases in the sRaw.

The results of the present murine study contrast with those observed in the cedar pollen-induced guinea pig allergic rhinitis study, in which guinea pigs produced a large amount of allergen-specific IgE after intranasal sensitizations with the pollen extract + Al(OH)<sub>3</sub> with the same dosage used in Group 1A.<sup>1,2</sup> Moreover, the sensitized guinea pigs clearly showed not only immediate sneezing but also biphasic nasal blockage after pollen challenges.<sup>2-6</sup> These observations suggest that mice are less sensitive to the pollen allergen than guinea pigs, and that guinea pigs may be susceptible to developing pollen-induced nasal blockage. On the other hand, the sneezing frequency

of sensitized-challenged mice decreased on the 13th challenge in comparison with the response on the 8th challenge. However, because sneezing was induced even after the 20th challenge with almost identical frequency to that on the 13th challenge (data not shown), sensitized-challenged mice may have not acquired tolerance to the antigen.

sRaw is measured by using a double-flow plethysmography that detects both the nasal and thoracic airflows.<sup>25</sup> The nasal flow lags behind the thoracic flow by several milliseconds, a delay which is affected by the airway resistance. Therefore, when airway obstruction occurs, the delay is expanded.<sup>26</sup> sRaw, therefore, can be calculated based on this theory.<sup>26</sup> Because the double-flow plethysmography allows us to longitudinally measure the airway mechanics without having to use any anesthesia or perform any surgical operations, it has been used as a reliable parameter for measuring airway resistance.<sup>2-6</sup> Since the sRaw reflects changes in both the upper and lower airways, we decreased the volume (2  $\mu$ l/nostril) of the intranasally instilled antigen suspensions or solution as much as possible in order to restrict the allergic site to the upper airway tissues. Thus, we believe that the changes in the sRaw in the present model reflect the nasal blockage response. Therefore, the lack of any increase in sRaw strongly indicates that nasal blockage was not induced even in Group D mice which were sensitized/challenged with Cry j 1.

Miyahara *et al.*<sup>15,16</sup> reported that when BALB/c mice sensitized by intraperitoneal administration of OVA were challenged by intranasal application of OVA 3 or 6 times, nasal blockage was immediately in-

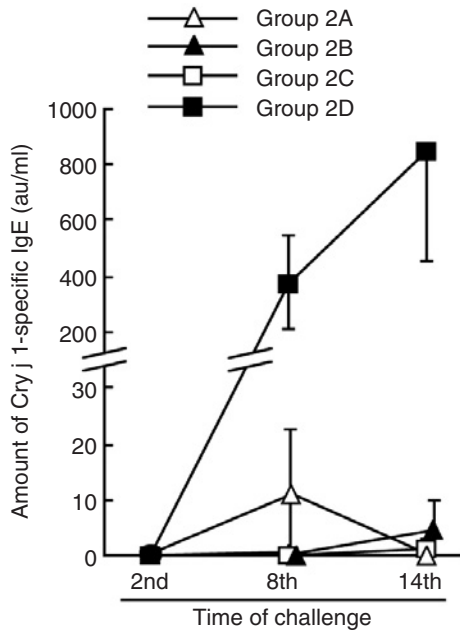


**Fig. 4** Time-course changes in specific airway resistance (sRaw) after the 1st (A, B, C and D), 5th (E, F, G and H) and 15th (I, J, K and L) challenges with either Japanese cedar pollen suspension (Groups 2A and 2C) or Cry j 1 (Groups 2B and 2D) in mice sensitized with either the pollen extract + Al(OH)<sub>3</sub> (Groups 2A and 2B) or Cry j 1 + Al(OH)<sub>3</sub> (Groups 2C and 2D). Each point represents the mean  $\pm$  S.E. of 5–10 animals.

duced and could be observed for up to 24 hours after the challenge. In their study, however, they measured the nasal airway resistance under artificial ventilation from the larynx toward the nostril in systemically anesthetized mice.<sup>15,16</sup> Therefore, several differences exist between the previous results and our current study, including (1) antigen (species and dosage), (2) mouse strain, (3) sensitization/challenge protocol and (4) methods for measurement of airway resistance. Since we could not determine the exact

reasons as to why we were not able to detect nasal blockage in our model, further studies are required in order to confirm the present results.

Although sneezing was induced in all of the sensitized groups, Cry j 1-specific IgE production only occurred after a challenge with Cry j 1 in Group 2D mice. In our guinea pig models of allergic rhinitis<sup>2-6</sup> and asthma,<sup>27-29</sup> both of which were developed by local and not systemic sensitization/challenge, incidences of allergic airway responses were induced



**Fig. 5** Time-course changes in amount of Cry j 1-specific IgE during challenges with either Japanese cedar pollen suspension (Groups 2A and 2C) or Cry j 1 (Groups 2B and 2D) in mice sensitized with either pollen extract + Al (OH)<sub>3</sub> (Groups 2A and 2B) or Cry j 1 + Al (OH)<sub>3</sub> (Groups 2C and 2D). Each point represents the mean  $\pm$  S.E. of 6–9 animals.

even when serum antigen-specific IgE had not fully increased.<sup>2,27</sup> In our models, animals were sensitized locally, as we believe that local sensitization is advantageous in that it provides an antigen invasion route that is identical to that seen in clinical settings. We believe this advantage outweighs the model's disadvantage of requiring a much longer time to fully sensitize experimental animals. Our present results suggest that local sensitization was established within the nasal tissues after multiple antigen applications which led to the diffusion of antigen-specific IgE into the circulation.

Overall, nasal blockage was not induced in the Japanese cedar pollen-induced allergic rhinitis model in B10.S mice, even in animals possessing high amounts of Cry j 1-specific IgE and showing signs of sneezing.

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