

Full Paper

## Effect of Oral Immunotherapy on Nasal Blockage in Experimental Allergic Rhinitis

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**Abstract.** We previously reported that when Japanese cedar pollen was prophylactically p.o. administered before a sensitization stage in a guinea-pig model of allergic rhinitis, pollen-induced nasal blockage was suppressed. In this study, we evaluated whether the oral immunotherapy is also effective when the pollen extract was administered starting from the day when the nasal blockage was clearly induced and whether the effectiveness continued after cessation of the immunotherapy. Sensitized animals were repeatedly challenged by pollen inhalation once every week. After the 7th challenge, the extract was orally administered twice a week until the 30th challenge. At the 11th challenge, the oral immunotherapy showed inhibition of the biphasic nasal blockage. The effectiveness was consistently observed during the immunotherapy until the 30th challenge. Furthermore, the increased nasal responsiveness to intranasal application of leukotriene D<sub>4</sub> was markedly suppressed by the immunotherapy. Interestingly, even after cessation of the therapy, inhibition of the nasal blockage was sustained for more than 2 months. Nevertheless, neither sneezing nor antigen-specific IgE antibody production was substantially influenced by the immunotherapy. In conclusion, Oral immunotherapy may be clinically useful for allergic nasal blockage. Mechanisms underlying the effectiveness may be associated with the hyporesponsiveness of the nasal mucosa to released mediators.

**Keywords:** allergic rhinitis, animal model, oral immunotherapy, nasal blockage, pollenosis

### Introduction

The best way for patients to relieve allergic rhinitis symptoms, such as sneezing, rhinorrhea, and nasal blockage in pollenosis, is the avoidance of pollen during normal daily activities. Since complete avoidance is difficult to achieve, several drugs have been used to treat allergic rhinitis. Sneezing and watery rhinorrhea have been shown to be suppressed by anti-histaminics (1–4). However, nasal blockage does not respond to these compounds and can only be relieved by  $\alpha$ -adrenoceptor stimulatory decongestants (5) and corticosteroids (6), which unfortunately exhibit tachyphylaxis during repetitive use over a short period (7) and other well-known side effects (8), respectively.

Since drug administration only provides symptomatic

therapy, specific immunotherapy is the sole way for developing true allergic disease treatments. Thus, many investigators have examined the effects of specific immunotherapies via the use of subcutaneous administrations of pollen antigens to relieve the symptoms of the disease (9). Although the effectiveness of subcutaneous immunotherapy has been established, it has also been pointed out that subcutaneous injections of antigen could potentially result in the occurrence of life-threatening anaphylactic reactions (10). However, as there have been few such incidences, clinical trials of oral immunotherapy in patients with allergic rhinitis have been conducted. The results obtained so far are conflicting and the efficaciousness of this method remains controversial (11). This controversy might be related to the limitations of the clinical experiments that have been performed.

In order to analyze the mechanisms of allergic rhinitis

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and to develop therapeutic drugs and methods, we have established an experimental allergic rhinitis model that is based on the use of repetitive inhalation challenges with Japanese cedar pollen as the antigen in sensitized guinea pigs (12–15). Nasal symptoms of the model are similar to those seen in allergic rhinitis patients and include the following: 1) production of antigen-specific anaphylactic antibodies in the serum (12, 13), 2) immediate induction of frequent sneezing after antigen provocation (13), 3) induction of biphasic nasal blockage in a reproducible manner after multiple pollen challenges (13), and 4) marked increases in nasal responsiveness to histamine (14) and leukotriene (LT) D<sub>4</sub> (15) with repeated challenges.

Using this model, we previously examined the effect of oral immunotherapy on the symptoms of allergic rhinitis (16). When twice a week oral administration of 1 and 100 mg of cedar pollen suspension/animal was started 2 weeks before the sensitization procedure, both doses potently suppressed the biphasic nasal blockage and increased nasal responsiveness to histamine and LTD<sub>4</sub> (16). In contrast, neither sneezing nor IgE production were affected in the same study. However, the protocol, in which there is administration of the allergen before the sensitization, does not exactly duplicate the clinical situation, as patients who are suffering from pollenosis and who need immunotherapy most likely have already been sensitized to the pollen before the procedure. Additionally in our previous study, 4 out of 10 guinea pigs died from complications of diarrhea during the course of repetitive administration of the 100-mg pollen suspension. This was possibly due to a cytotoxic effect on the digestive tract caused by the resin or some other agent that was contained in the pollen (16).

In this study, an extract from 1 mg of the pollen was orally administered after the allergen-induced biphasic nasal blockage induction at the 7th inhalation challenge. Additionally, the magnitudes of the nasal blockages induced by the allergen and LTD<sub>4</sub>, sneezing, and allergen-specific IgE production were observed during the immunotherapy until the 30th challenge and compared with that observed in the phosphate-buffered saline (PBS)-treated control group. Oral administration of the pollen ceased at the 30th challenge and was followed by observation of the subjects for 2 additional months to determine whether there was sustainment of the immunotherapy effect during this time period.

## Materials and Methods

### Animals

Male, 3-week-old Hartley guinea pigs were purchased

from Japan SLC (Hamamatsu). The animals were housed in an air-conditioned room at a temperature of  $23 \pm 1^\circ\text{C}$  and  $60 \pm 10\%$  humidity with the lights on from 8:00 a.m. to 8:00 p.m. Animals were fed a standard laboratory diet and given water ad libitum. The first sensitization was started 2 weeks after arrival of the guinea pigs.

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. 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 previously described (17).

Cedar pollen extracts used for sensitization were prepared as follows: The pollen was suspended in PBS at 100 mg/ml followed by vigorous stirring at  $4^\circ\text{C}$  for 48 h. After the suspension was centrifuged ( $4^\circ\text{C}$ ,  $1,700 \times g$ , 15 min), the supernatant was collected. Protein concentration in the supernatant was measured by the method of Bensadoun and Weinstein (18). The extracts were combined with Al(OH)<sub>3</sub> suspension to be a concentration of 100  $\mu\text{g}$  protein/100 mg Al(OH)<sub>3</sub> in 1 ml of physiological saline.

For preparing pollen extracts used for oral immunotherapy, the pollen suspension (100 mg/ml) was centrifuged ( $4^\circ\text{C}$ ,  $31,000 \times g$ , 30 min) after stirring for 48 h. The supernatant collected was used for oral administration at a concentration of 1 mg pollen-derived extract /2 ml.

### Sensitization and challenge

As previously described (13), twice a day for 7 days, guinea pigs were sensitized by bilateral intranasal instillation of cedar pollen extracts adsorbed onto Al(OH)<sub>3</sub> gel (0.3  $\mu\text{g}$  protein/0.3 mg Al(OH)<sub>3</sub>) at a volume of 3  $\mu\text{l}$ /each nostril. To prevent the rapid elimination of the antigen by ciliary movement, prior to each sensitization, the upper airway mucosal surface was anesthetized by using a 2-min inhalation of a mist of 4% lidocaine hydrochloride solution (Fujisawa Pharm. Co., Osaka) that was generated by an ultrasonic nebulizer (NE-U12; Omron, Osaka). The sensitized animals were then intranasally challenged once every week until the 40th challenge by inhalation of cedar pollen using a hand-made inhalation apparatus (12), which was designed to allow quantitative inhalation of pollen. The apparatus was loaded with 3 mg of pollen and positioned in one nostril of the conscious guinea pig for 1 min so that inhalation of approximately 1.8 mg of the pollen occurred by spontaneous breathing. During

the inhalation, the other nostril was plugged with a finger. The procedure was repeated a second time for the other nostril. As a negative control, a sensitized–non-challenged group was prepared for the experiments that assessed nasal responsiveness to LTD<sub>4</sub>.

#### *Oral administration of the cedar pollen extract for immunotherapy*

As previously reported (13), the 7th pollen challenge stably induced the allergic nasal symptoms of biphasic nasal blockage and sneezing (Fig. 1). Starting on the 1st day after the 7th challenge until 1 day before the 30th challenge, 1 mg of pollen-derived extract/animal was orally administered using a polyvinyl chloride tube (Atom indwelling feeding tube for infant, 4Fr; Atom, Tokyo) without any antacids twice a week during this period. On the days of the pollen inhalation challenge, no oral administration for immunotherapy was performed.

#### *Counting of sneezing frequency*

Sneezing frequency during 0–1 h after the pollen inhalation challenge was counted.

#### *Measurement of specific airway resistance (sRaw)*

sRaw was used as an indication of nasal blockage and was measured by a two-chambered, double-flow plethysmograph system according to the method of

Pennock et al. (19). Briefly, the animal was placed with its neck extending through the partition of a two-chambered box. sRaw was measured with the data analyzer Pulmos-I (MIPS, Osaka) and a PC 9801 FA computer (NEC, Tokyo) once there was detection of the airflow by the sensors located in both the front and rear chambers.

#### *Measurement of nasal responsiveness to LTD<sub>4</sub>*

Two days after the pollen inhalation challenges, nasal responsiveness to intranasally instilled LTD<sub>4</sub> was measured as previously described (15). At 20-min intervals, increasing doses (10 µl/each nostril) of the LTD<sub>4</sub> solution (10<sup>-8</sup> and 10<sup>-6</sup> M) were bilaterally applied. sRaw was measured 10 min after each of the respective agonist doses.

#### *Measurement of Cryj 1- and Cryj 2-specific IgE antibodies in sera*

Cryj 1- and Cryj 2-specific IgE antibodies were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Guinea pig IgE ELISA MARUPI; Dainippon Pharmaceutical Co., Osaka) using blood samples collected 1 day before the inhalation challenges. Because this kit is for the measurement of guinea-pig total IgE, we modified the method provided by the manufacturer to measure Cryj 1- and Cryj 2-specific IgEs. Diluted sera were added into the wells of a microtiter plate pre-coated with an anti-guinea-pig IgE antibody, and then the plate was incubated for 1 h at 15–25°C. After washing, 100 ng/ml of either biotinylated Cryj 1 or Cryj 2 (Asahi Food & Healthcare Co., Tokyo) was added at a volume of 100 µl/well, followed by incubation for 1 h at 15–25°C. The plate was washed, and then avidin-horseradish peroxidase conjugate (BD Pharmingen, San Diego, CA, USA) was added. Following incubation for 30 min and a subsequent washing, substrate was added, and the enzyme reaction was developed for 30 min at 15–25°C. The reaction was stopped with a stop solution and absorbance values were measured at 450 nm.

Values for Cryj 1- and Cryj 2-specific IgE levels in tested sera were expressed in arbitrary units relative to the value of a pooled standard serum from the sensitized-challenged guinea pigs. The standard serum was prepared by i.p. injection of the pollen extract adsorbed onto Al(OH)<sub>3</sub> once every week for a total of 9 times in naïve guinea pigs. The sera were collected 2 weeks after the last sensitization, followed by a subsequent pooling of all sera obtained. Cryj 1- and Cryj 2-specific IgE titers of the pooled serum were regarded as 1000 AU/ml.

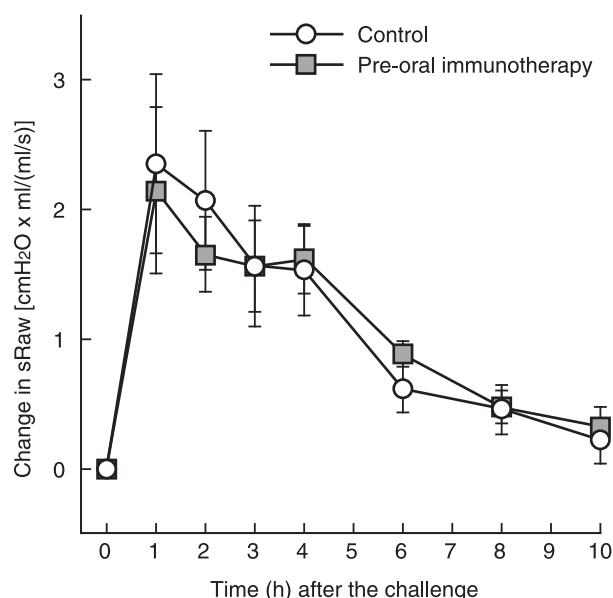


**Fig. 1.** Time-course change in specific airway resistance (sRaw) after the 7th inhalation challenge with Japanese cedar pollen in sensitized guinea pigs. Based on the results at the 7th challenge, 16 animals were divided into 2 groups, control and oral immunotherapy, respectively. Each point represents the mean  $\pm$  S.E.M. of 8 animals.

### Statistical analyses

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

## Results

### Influence on the biphasic nasal blockage

The 7th pollen inhalation challenge stably induced biphasic nasal blockage (Fig. 1), as has been previously reported (13). Starting on the 1st day after the 7th challenge, the pollen extract was orally administered twice a week. Figure 2 shows the effect of oral immunotherapy on the biphasic nasal blockage at the 9th–30th inhalation challenges. Early (EPR) and late phase response (LPR) are expressed as the area under the response curve (AUC) for the changes in sRaw at 0–2 h and 3–6 h after challenge, respectively. The oral immunotherapy significantly inhibited both the EPR and LPR at the 11th challenge, which is the 4th week after the start of the oral administration. The effectiveness was sustained at the 16th and 30th challenges during the immunotherapy (Fig. 2).

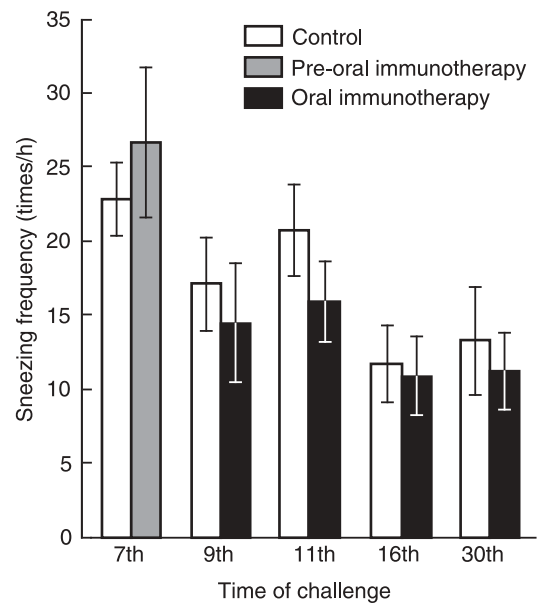


**Fig. 3.** Effect of the oral immunotherapy on the occurrence of sneezing induced by the 9th–30th inhalation challenges with Japanese cedar pollen in sensitized guinea pigs. Based on the results at the 7th challenge, 16 animals were divided into 2 groups, control and oral immunotherapy, respectively. The extract derived from 1 mg pollen was orally administered twice a week from the 1st day after the 7th challenge to the 30th challenge. Sneezing frequency was counted during 0–1 h after each challenge. Each column represents the mean  $\pm$  S.E.M. of 8 animals.

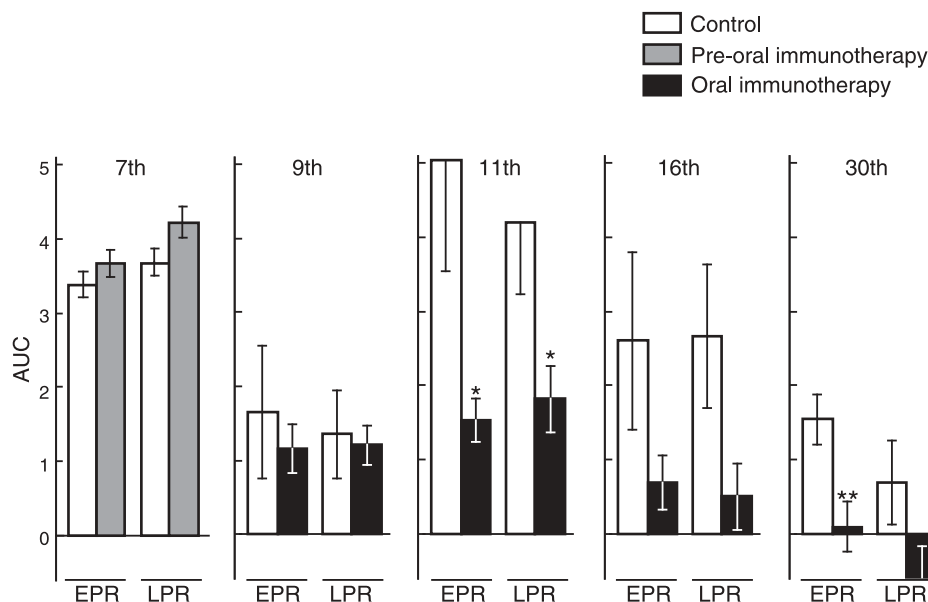


**Fig. 2.** Effect of the oral immunotherapy on early (EPR) and late phase response (LPR) of the biphasic nasal blockage induced by the 9th–30th inhalation challenges with Japanese cedar pollen in sensitized guinea pigs. The extract derived from 1 mg pollen was orally administered twice a week from the 1st day after the 7th challenge to the 30th challenge. EPR and LPR are expressed as the area under the response curve (AUC) for the change in the specific airway resistance at 0–2 h and 3–6 h after the challenges, respectively. Each column represents the mean  $\pm$  S.E.M. of 8 animals. \* $P$ <0.05 and \*\* $P$ <0.01 vs control.

### Influence on sneezing

As previously reported (13), most sneezing was induced within 0–1 h after the respective pollen inhalation challenges, with values of 10–25 times/h. In contrast to the effects seen for nasal blockage, the oral immunotherapy did not substantially affect the occurrence of the sneezing induced by the 9th–30th inhalation challenges (Fig. 3).

### Influence on *Cry j 1*- and *Cry j 2*-specific IgE production

Amounts of both *Cry j 1*- and *Cry j 2*-specific IgEs increased in the serum after the multiple pollen inhalation challenges during the 7th–29th inhalation challenges. The oral immunotherapy produced no effect on these IgE productions (Fig. 4).

### Influence on nasal responsiveness to intranasal instillation of LTD<sub>4</sub>

Consistent with our previous finding (15), nasal responsiveness to LTD<sub>4</sub> for the control group was considerably marked compared with that of the sensitized–non-challenged guinea pig on the 2nd day after the challenges (Fig. 5). The development of the nasal hyperresponsiveness at the 16th and 30th inhalation challenges was almost completely suppressed by oral immunotherapy (Fig. 5).

### Sustained effect of the immunotherapy after ceasing administration

To evaluate whether the effectiveness of the immunotherapy on the symptoms of allergic rhinitis is sustained

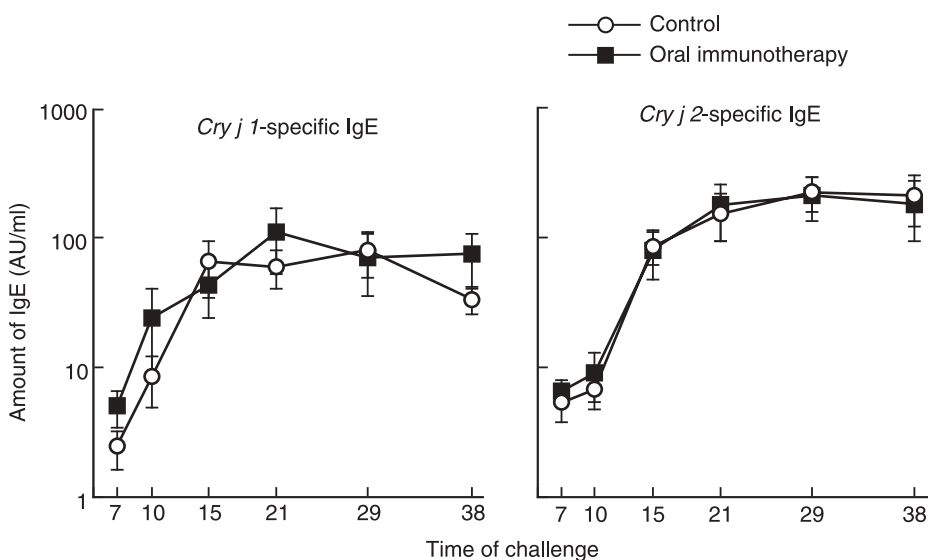


**Fig. 4.** Effect of the oral immunotherapy on the production of *Cry j 1*- and *Cry j 2*-specific IgEs during the 7th–38th inhalation challenges with Japanese cedar pollen in sensitized guinea pigs. The extract derived from 1 mg pollen was orally administered twice a week from the 1st day after the 7th challenge to the 30th challenge. Sera were drawn 1 day before each challenge. Each point represents the mean  $\pm$  S.E.M. of 8 animals.

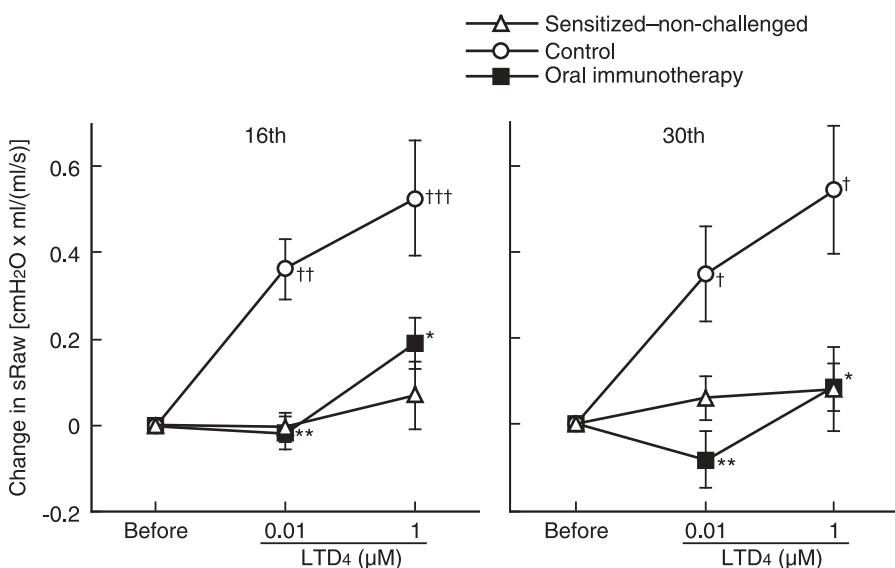


**Fig. 5.** Effect of the oral immunotherapy on the increase of nasal responsiveness to leukotriene (LT) D<sub>4</sub> after the 16th and 30th inhalation challenges with Japanese cedar pollen in sensitized guinea pigs. The extract derived from 1 mg pollen/animal was orally administered twice a week from the 1st day after the 7th challenge to the 30th challenge. The nasal responsiveness to intranasal instillation of 0.01 and 1  $\mu$ M LTD<sub>4</sub> was assessed 2 days after the challenges. Each point represents the mean  $\pm$  S.E.M. of 8 animals. \* $P$ <0.05 and \*\* $P$ <0.01 vs control; † $P$ <0.05, †† $P$ <0.01, and ††† $P$ <0.001 vs sensitized–non-challenged group.

after ceasing administration of the extract, we stopped the oral administration 1 day after the 30th challenge and then observed the nasal blockage and hyperresponsiveness at the 35th and 39th challenges. The effectiveness was consistently sustained even at these challenges (Fig. 6). On the other hand, sneezing at the 39th challenge was still unaffected after the immunotherapy. Although the number of sneezings in the post immunotherapy group at the 35th challenge was smaller compared to the control, the degree was not statistically significant (Fig. 6). In addition, the level of neither *Cry j 1*- nor *Cry j 2*-specific IgE was affected even after the immunotherapy at the 39th challenge (Fig. 4).

## Discussion

In clinical situations, immunotherapy for pollenosis is conducted for symptomatic or asymptomatic patients who have already been sensitized to the specific pollen. In contrast to our previous schedule (16), the oral immunotherapy in the present study was started one day after the 7th challenge, the point where not only sneezing but also biphasic nasal blockage is consistently induced. Results indicated that biphasic nasal blockage, consisting of both EPR and LPR, was significantly suppressed by the oral immunotherapy at the 11th challenge (4 weeks after the start of the oral administra-

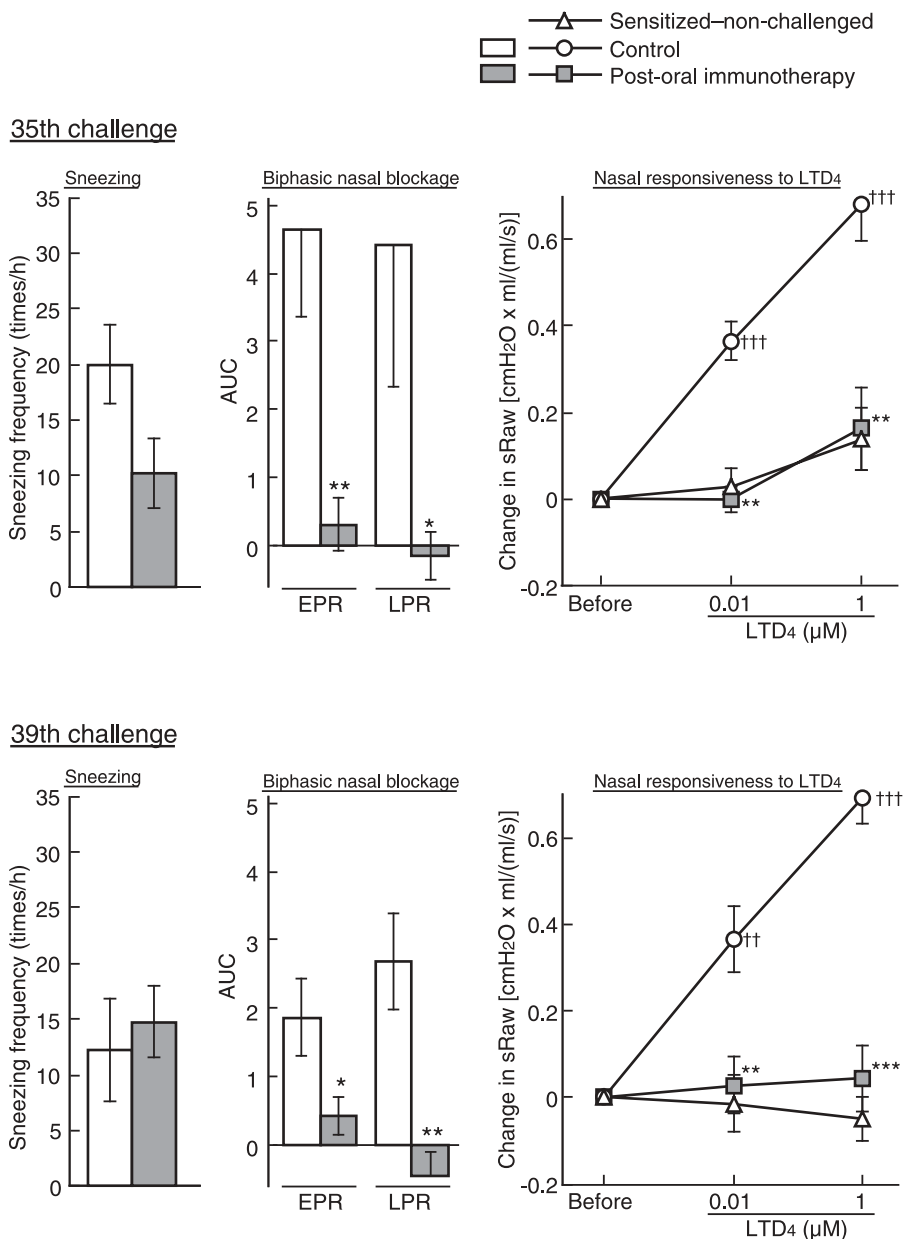


**Fig. 6.** Sustained effect of the oral immunotherapy on the sneezing, early (EPR) and late phase response (LPR) of the biphasic nasal blockage, and the increase of nasal responsiveness to leukotriene (LT)  $D_4$  after the 35th and 39th inhalation challenges with Japanese cedar pollen in sensitized guinea pigs. The extract derived from 1 mg pollen/animal was orally administered twice a week from the 1st day after the 7th challenge to the 30th challenge. After ceasing the oral immunotherapy on the 1st day after the 30th challenge, nasal blockage induced by the inhalation of pollen was assessed at the 35th and 39th challenges. In addition, the nasal responsiveness to intranasal instillation of 0.01 and 1  $\mu$ M LTD $_4$  was assessed 2 days after these challenges. EPR and LPR are expressed as the area under the response curve (AUC) for changes in specific airway resistance at 0–2 h and 3–6 h after the challenges, respectively. Each column and point represents the mean  $\pm$  S.E.M. of 8 animals. \* $P$ <0.05, \*\* $P$ <0.01, and \*\*\* $P$ <0.001 vs control; †† $P$ <0.01 and ††† $P$ <0.001 vs sensitized-non-challenged group.

tion). Inhibition or the tendency for suppression was constantly seen at the 16th and 30th challenges. On the other hand, neither sneezing nor *Cryj 1*- and *Cryj 2*-specific IgE production was influenced by the immunotherapy. At the 16th and 30th challenges, nasal responsiveness to intranasal application of LTD<sub>4</sub> was markedly increased in the control animals, whereas there was no increase of the responsiveness produced in guinea pigs treated with the immunotherapy. In addition, repetitive oral administration of 1 mg pollen-derived extract showed no visible symptom in the guinea pigs sensitized with the pollen.

The present results are not substantially different from our previous study that examined the oral administration of the pollen 2 weeks prior to the sensitization with the pollen extract adsorbed on Al(OH)<sub>3</sub> (16), thereby suggesting that the oral immunotherapy is effective even in symptomatic patients during the pollen season. However, because the effectiveness was observed at the 11th but not the 9th challenge, this indicates that it will take approximately one month to achieve the immunotherapy.

Previous clinical studies of oral immunotherapy have yielded conflicting results (11). A 1-year oral administration of a high dose of grass pollen to patients with hay fever failed to produce any beneficial change in either the clinical symptoms or the serum antigen-specific IgE levels (20). On the other hand, when patients with allergic rhinitis to short ragweed were treated orally with encapsulated short ragweed extract for 6 weeks followed by a nasal antigen challenge, the nasal congestion was significantly suppressed, even though the sneezing, nasal secretion, and the serum antigen-specific IgE antibody levels were not substantially attenuated (21). Since nasal blockage is apparently induced mainly by nasal congestion, our present findings correspond well with those of the latter report (21).

We have previously reported that sneezing induced within 1 h after challenge was almost completely suppressed by treatment with anti-histaminics (22, 23). This indicates that histamine released from mast cells immediately after the pollen inhalation plays a major role in the occurrence of sneezing. Nevertheless, the oral immunotherapy produced no inhibition on the induction of sneezing. In addition, the increase of *Cryj 1*- and *Cryj 2*-specific IgE levels was not altered by the immunotherapy. These findings are similar to both those in our previous pre-treatment experiment (16), in which the oral administration was started before the sensitization procedure, and to the other clinical studies that have evaluated oral immunotherapy (20, 21). These results indicate that oral immunotherapy has no inhibitory effect on the IgE-mediated mast cell acti-

vation at the nasal mucosa.

However, mechanisms that can explain the effectiveness of the oral immunotherapy on the biphasic nasal blockage other than by inhibition of IgE production and mast cell activation are not clear at the present. On the other hand, the current results revealed that the increased nasal responsiveness to LTD<sub>4</sub> was not induced in the immunotherapy group. Thus, the inhibition of the biphasic nasal blockage by the immunotherapy may be due to the suppression on the development of nasal hyperresponsiveness. It has been indicated that nasal blockage is induced mainly by both dilatation of nasal blood vessels and increased vascular permeability (24). We have demonstrated that both the antigen- (25) and the LTD<sub>4</sub>- (15) induced nasal blockages in the sensitized guinea pig were largely inhibited by a vasoconstrictive  $\alpha$ -adrenergic agonist, naphazoline. Furthermore, we found that constitutive nitric oxide (NO) synthase-derived NO is a major mediator in the nasal blockage (15, 25), suggesting that NO plays an important role in both the vasodilatation and extravascular leakage of plasma components. In addition, nasal hyperresponsiveness in our model can be observed with not only LTD<sub>4</sub> but also with histamine (14) and a thromboxane A<sub>2</sub> mimetic, U-46619 (Yamasaki et al., unpublished observation). Therefore, the oral immunotherapy should decrease responsiveness of the target organ, i.e., the nasal blood vessel, to the various chemical mediators that are released. However, the reason why exposure of the gastrointestinal mucosa to the specific allergen leads to hyporesponsiveness needs to be elucidated in the future. At present, there is no literature suggesting that there may be a relationship between these mucosal immune systems with regard to suppression of allergic symptoms.

To assess whether the efficaciousness of the immunotherapy was maintained after the cessation of the oral administration of the extract, we stopped the administration at the 30th challenge. We observed that occurrences of the biphasic nasal blockage were present for 9 more weeks. Interestingly, the magnitudes of both the EPR and LPR of the nasal blockage were consistently smaller in the immunotherapy group. In line with the results for the biphasic nasal blockage, the development of nasal hyperresponsiveness was also completely suppressed by the immunotherapy. These findings further suggest that the hyporesponsiveness of the nasal mucosa may be associated with the inhibition of the biphasic nasal blockage by the immunotherapy and that oral immunotherapy might be effective even after cessation of the therapy in clinical settings.

In conclusion, when the pollen extract was orally administered twice a week starting from the point at

which the guinea pigs were sensitized and clearly exhibited biphasic nasal blockage, the biphasic nasal blockage but not the sneezing induced by subsequent pollen inhalation challenges was effectively suppressed. This inhibition of the nasal blockage was not related to serum antigen-specific IgE levels. The effectiveness of the therapy could be due to nasal hyporesponsiveness to the chemical mediators released.

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