Efficacy of subcutaneous immunotherapy in a mouse model of Humulus pollen-induced allergic asthma

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Abstract: Objective: The aim of this study was to evaluate the therapeutic effect of subcutaneous injection of high-dose of Humulus pollen allergen on a mouse model of allergic asthma. Method: Humulus pollen-sensitized mice received either regular subcutaneous injections of Humulus pollen allergen extract or control treatment were sacrificed. Airway responsiveness, pulmonary inflammatory cell infiltration, serum cytokine secretion and serum immunoglobulin E (IgE) and G2a (IgG2a) levels were measured. Result: Our results showed that subcutaneous immunotherapy with high-dose of Humulus pollen allergen could obviously relieve the symptoms and improve the pulmonary function in mice with allergic asthma. It also elevated serum IgE and IgG level compared with model group. The Humulus pollen allergens could be identified in serum and the molecular weight of serum proteins that had the maximum binding to allergen were ~25 kDa and ~38 kDa in 7 cases (70%) and 6 cases (60%), respectively. Conclusion: Subcutaneous immunotherapy (high-dose) could effectively alleviate airway hyperresponsiveness and reduce airway inflammatory infiltration in allergic asthmatic mice.

Keywords: Immunotherapy, humulus pollen, allergic asthma, immunoglobulin

Introduction

Asthma is featured by chronic inflammatory response in the airways, with allergic asthma being the most common type. Allergens can induce or exacerbate allergic asthma through an immunoglobulin E (IgE)-dependent immune response. Humulus pollen is an important allergen responsible for the occurrence and development of fall pollen-induced allergic asthma in China [1]. Humulus scandens is mainly distributed in subtropical and temperate zones in Northern Hemisphere. In China, it is mainly distributed in the Northeast, Northern, Central and Southern, Southwest, Shanxi and Gansu regions. Yin reported that Humulus pollen was one of the determinant causes of seasonal allergic rhinitis and asthma [2]. Allergen-specific immunotherapy is currently the only etiological treatment for allergic asthma [3], probably through changing the natural course of the disease, stopping the development of asthma and preventing allergic sensitization to new allergens [4]. At present, Humulus pollen allergen-specific immunotherapy is widely used in clinical practice, but it has certain defects such as low therapeutic concentration, long treatment course and poor patient compliance. In addition, there is no relevant research evaluating the efficacy of specific immunotherapy with high-dose and short-term treatment.

Sun et al established a mouse model of Humulus pollen-induced pulmonary inflammation by subcutaneous injection and intranasal instillation of Humulus pollen allergen [5]. This model is useful to investigate the etiopathology of the disease and to find treatments. In this study, a similar animal model of Humulus pollen-induced allergic asthma was established and pulmonary function and allergen specific antibodies in mice were detected. Our study aimed to evaluate the efficacy of high-dose allergen-specific immunotherapy.

Materials and methods

Experimental animals

60 female Balb/c mice (6-8 weeks old, SPF) were provided by the Military Medical Science
Subcutaneous immunotherapy for allergic asthma

Academy of the PLA, China. Animals were housed in a temperature and humidity-controlled room in the Laboratory Animal Center of Peking Union Medical College Hospital with free access to sterile diet. The temperature was controlled at 22-25°C and the humidity level was maintained at 40-60%. All the animal procedures were approved by the ethics committee of Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College.

Allergen

Humulus pollen was collected from the agricultural areas around Beijing. Liquid nitrogen grinding was used to destroy the cell walls. Pollens were defatted by acetone, extracted with phosphate buffer, filtered through 0.2 μm pore-sized filter membranes, dialyzed with de-ionized water, then freeze-dried and kept in vials. The concentration of 200 μL redissolution was 7.384 μg/μL (total protein 1.477 mg per bottle).

Experimental procedure

Mice were randomly divided into control, model and immunotherapy groups (20 rats in each group). Mice in the model group were intraperitoneally injected with Humulus pollen extract on the 1st, 7th and 14th day, respectively, and were nasally instilled extract on the 21st and 22nd day. Allergen-specific immunotherapy was performed by subcutaneous injections with Humulus pollen extract from the 15th to 23rd day. Mice in the control group were sensitized and stimulated in the same way as model group except for using the same volume of normal saline instead of crude extract of Humulus pollen. 10 mice in each group were anesthetized with intraperitoneal injection of 10% chloral hydrate 24 h after last stimulation. Blood samples were drawn from the orbital arteries and serum was collected by centrifuging. Animals were sacrificed by cervical dislocation. The left upper pulmonary lobe was isolated and fixed with formalin, and stained with Hematoxylin-Eosin (HE) and Alcian blue-Period acid Schiff (AB-PAS) staining.

General conditions of the mice were observed including drooping of the head, decreased activity, clustering, piloerection, tachypnea, hunched back, forelimb flexion and allergic conjunctivitis. The frequencies of the above symptoms were compared among groups.

Assessment of airway hyperresponsiveness

10 mice were selected from each group to assess pulmonary function. In order to avoid the influence of acetyl methylcholine chloride (Mch) on other experimental indices, mice that underwent pulmonary function assessment were spared from following sampling.

10 mice were selected from each group to perform aerosol inhalation of methacholine (0.938, 1.875, 3.75, 7.5, 15 and 30 mg/mL) for 2 min 24 h after the last allergen stimulation. Pulmonary function index at the corresponding dose was determined by the average enhanced pause (Penh) value within 3 minutes after the inhalation. Penh was calculated following the formula: Penh = [(Te/Tr-1) × (PEF/PIF)]. Te is the expiration time, Tr is the relaxation time, PEF is the peak expiratory flow rate and PIF is the peak inspiratory flow rate multiplied by the constant coefficient 0.67. Relaxation time is...
defined when the pressure in the plethysmograph changes from the maximum value to the minimum value (the percentage of the maximum value, usually 40%).

Biochemical measurement

Total IgE detection kit (ab157718, Abcam) was used to detect total IgE and experiments were performed in strict accordance with the manufacturer’s instructions.

The experimental procedure for IgE assessment included antigen coating overnight at 4°C, blocked with 1% BSA at 37°C for 2 h, adding diluted test serum at 37°C for 5 h, incubating with enzyme-labeled anti-IgE secondary antibody (ab99574, Abcam) at 37°C for 5 h, coloring, terminating reaction, and reading plate.

The experimental procedure for IgG1 included antigen coating overnight at 4°C, blocked with 1% BSA at 37°C for 2 h, adding diluted test serum at room temperature for 1 h, incubating with enzyme-labeled anti-IgG1 secondary antibody (ab97240, Abcam) at room temperature for 50 min, coloring, terminating reaction, reading plate. Detection of antigen-specific IgG2a (specific IgG2a, sIgG2a) was the same as serum IgG1 with anti-IgG2a secondary antibody (ab97245, Abcam).

Measurements of cytokines

The serum T helper (Th) 1 cytokine interleukin (IL)-12p70, interferon (IFN)-γ, Th2 cytokine IL-5 and IL-13 were detected by commercial kits (ab100699, Abcam; BMS606, eBioscience; ab100711, Abcam and BMS601, eBioscience, respectively).

HE and AB-PAS staining

HE and AB-PAS staining were completed by the Department of Pathology at Institute of Basic Medical Sciences of Peking Union Medical College. The inflammatory pathological changes of mouse airways were evaluated with the Cosio score [6] and the degree of airway mucus secretion was assessed using MOR (ratio of mucus area to bronchial area) [7] (Table 1).
Subcutaneous immunotherapy for allergic asthma

Table 2. Comparison of symptom frequencies in the model and immunotherapy groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Model</th>
<th>Immunotherapy</th>
<th>Fisher’s test</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+</td>
<td>- Total</td>
<td>+</td>
</tr>
<tr>
<td>Head drooping</td>
<td>18</td>
<td>2 20 4 16 20</td>
<td>4</td>
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<tr>
<td>Decreased activity</td>
<td>18</td>
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<tr>
<td>Clustering</td>
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</tr>
<tr>
<td>Piloerection</td>
<td>20</td>
<td>0 20 4 16 20</td>
<td>4</td>
</tr>
<tr>
<td>Nodding breathing</td>
<td>16</td>
<td>4 20 0 20 20</td>
<td>2</td>
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<tr>
<td>Hunched back</td>
<td>14</td>
<td>6 20 0 20 20</td>
<td>6</td>
</tr>
<tr>
<td>Forelimb flexion</td>
<td>14</td>
<td>6 20 0 20 20</td>
<td>6</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>16</td>
<td>4 20 6 14 20</td>
<td>4</td>
</tr>
</tbody>
</table>

Pearson test
χ² P
Total 136 24 160 26 134 160 151.274 0.0000

Table 3. Comparison of symptom frequencies in the immunotherapy and control groups

<table>
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<tr>
<th>Variables</th>
<th>Immunotherapy</th>
<th>Control</th>
<th>Fisher’s test</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>- Total</td>
<td>+</td>
</tr>
<tr>
<td>Head drooping</td>
<td>4</td>
<td>16 20 0</td>
<td>20 20 20</td>
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<tr>
<td>Decreased activity</td>
<td>6</td>
<td>14 20 0</td>
<td>20 20 20</td>
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<tr>
<td>Piloerection</td>
<td>4</td>
<td>16 20 0</td>
<td>20 20 20</td>
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<tr>
<td>Nodding breathing</td>
<td>0</td>
<td>20 20 0</td>
<td>20 20 20</td>
</tr>
<tr>
<td>Hunched back</td>
<td>0</td>
<td>20 20 0</td>
<td>20 20 20</td>
</tr>
<tr>
<td>Forelimb flexion</td>
<td>0</td>
<td>20 20 0</td>
<td>20 20 20</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>6</td>
<td>14 20 0</td>
<td>20 20 20</td>
</tr>
</tbody>
</table>

Pearson test
χ² P
Total 26 134 160 0 160 160 28.299 0.0000

Table 4. Marginal mean* of Penh

<table>
<thead>
<tr>
<th>Groups</th>
<th>Means</th>
<th>Standard error</th>
<th>95% confidence interval</th>
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<td></td>
<td>Minimum</td>
</tr>
<tr>
<td>Control</td>
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<td>.135</td>
<td>0.885</td>
</tr>
<tr>
<td>Model</td>
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<td>.136</td>
<td>4.688</td>
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<tr>
<td>Immunotherapy</td>
<td>2.609</td>
<td>.135</td>
<td>2.342</td>
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</table>

*Marginal Mean: The Penh’s marginal mean in this study refers to the Penh’s value after removing the influence of Mch. *The covariates appearing in this model are evaluated with the following values: Mch concentration 8.33426 mg/ml.

Immunoblotting

20 μL crude extract of Humulus pollen (1 μg/μL) was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (stacking gel: 80 V for 30 min; separating gel: 150 V for 150 min), Coomassie brilliant blue staining and decolorization. For the immunoblotting, the gel underwent transmembrane at 150 mA for 60 min and incubated with primary antibody (diluted with 5% milk at the ratio of 1:4) at room temperature for 2 h, followed by incubation with anti-IgE secondary antibody (ab995-74, Abcam, diluted with 5% milk at the ratio of 1:1000) at room temperature for 2 h.

Statistical analysis

Frequency of symptoms was tested by Chi-square test. The difference in the pulmonary function indices was evaluated by covariance analysis and the difference in the levels of antibodies and cytokines was analyzed with variance analysis. Pairwise comparison was made using LSD method (with homogeneity of variance) and Bonferroni method (with heterogeneity of variance) if the difference was significant. The Mann-Whitney U test was used to compare the pathological scores and the Bonferroni method was used to correct the P value. A P value < 0.05 was considered significant difference.

Results

General conditions

Allergic symptoms, such as drooping of the head, clustering, decreased activity, piloerection, nodding breathing, hunched back, forelimb flexion and allergic conjunctivitis were observed in all mice, whereas no symptom was observed in the control group (Figure 2A). The frequency of each symptom in the immunotherapy group was significantly lower than that in the model group (P < 0.05 for 8 indices) (Table 2). However, some symptoms of the mice in the immu-
Subcutaneous immunotherapy for allergic asthma

Immunotherapy reduced pulmonary index of Penh

The Penh value gradually increased with the increase of Mch in a dose-dependent manner in all three groups (Figure 2B). Under each Mch concentration, the Penh value of the model group was significantly higher than that of the control group ($P < 0.05$ under each concentration) and it was significantly lower in immunotherapy group than that in model group ($P < 0.05$ under each concentration), indicating that high-dose and short-course immunotherapy could improve the pulmonary function in asthmatic mice. There were significant differences in the marginal value of Penh among three groups when Mch concentration was 8.33 mg/mL ($P = 0.000$; Table 4).

Histopathological changes

Local mucous epithelial shedding, basement membrane thickening, increase in goblet cell numbers, hypertrophy and hyperplasia of vascular smooth muscle cells, infiltrations of eosinophils, lymphocytes and other inflammatory cells in the vascular wall and mucus plugs in the pulmonary alveoli could be observed in the airways in model group (Figure 3). Compared with the model group, the goblet cell proliferation, down of cilia and the mucus secretion score were significantly improved in immunotherapy group ($P < 0.01$). There was a tendency that the degree of eosinophil infiltration was less than that in model group, but the score

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Table 5. Mann-Whitney U test for the pulmonary pathological scores in the model and immunotherapy groups

<table>
<thead>
<tr>
<th>Inflammatory indices</th>
<th>Standardized test statistics (Z)</th>
<th>$P$ value</th>
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</thead>
<tbody>
<tr>
<td>Lymphocytic infiltration of the tracheal wall</td>
<td>-0.576</td>
<td>0.631</td>
</tr>
<tr>
<td>Neutrophil infiltration of the tracheal wall</td>
<td>0.798</td>
<td>0.425</td>
</tr>
<tr>
<td>Eosinophil infiltration of the tracheal wall</td>
<td>-1.990</td>
<td>0.047</td>
</tr>
<tr>
<td>Airway wall smooth muscle cell hyperplasia</td>
<td>-0.773</td>
<td>0.439</td>
</tr>
<tr>
<td>Tracheal epithelium goblet cell hyperplasia</td>
<td>-3.170</td>
<td>0.002</td>
</tr>
<tr>
<td>Tracheal cilia down and absence</td>
<td>-3.502</td>
<td>0.000</td>
</tr>
<tr>
<td>Mucous MOR</td>
<td>-2.894</td>
<td>0.004</td>
</tr>
</tbody>
</table>

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Figure 3. Airway inflammatory cell infiltration and alveolar mucus secretion in three groups (20 x). A. HE staining; Characteristics like local mucous epithelial shedding, basement membrane thickening, increase in goblet cell numbers, hypertrophy and hyperplasia of vascular smooth muscle cells, infiltrations of eosinophils, lymphocytes and other inflammatory cells in the vascular wall and mucus plugs in the pulmonary alveoli could be observed in the airways in model group; B. AB-PAS staining. Mucus suppository was found in the alveolar cavity of the model group, and the mucus suppository in the immunotherapy group was less than that in the model group.

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Immunotherapy group were not completely relieved compared to the control group ($P < 0.05$ for three indices) (Table 3).
Subcutaneous immunotherapy for allergic asthma

showed no significant difference (P = 0.047; Table 5).

*Immunotherapy elevated serum IgE and IgG*

The serum tlgE level was significantly higher in model group than in control group (P < 0.05), and it was significantly higher in immunotherapy group than in model group (P < 0.05; Figure 4A). The serum slgE level in the model group was significantly higher than in the control group (P < 0.05), but was not statistically different when compared with the immunotherapy group (P > 0.05; Figure 4B). The serum levels of slgG1 and slgG2a were significantly higher in the model group than in the control group (P < 0.05), and they were significantly higher in the immunotherapy group compared with the model group (P < 0.05; Figure 4B).

*Immunotherapy did not affect serum cytokines*

The level of Th1 type cytokine IL-12 in model group was significantly lower than that in control group (P < 0.05), but was not significantly different compared to immunotherapy group (P = 0.10; Figure 5).

The expression level of Th2 type cytokines IL-5 and IL-13 in model group was significantly higher than those in control group (P < 0.05), but were not significantly different compared to the immunotherapy group (P > 0.05; Figure 5).

*Protein identification*

The molecular weight of major proteins was ~80, ~70, ~38, ~30, ~26, ~15, and ~10 kDa, respectively, in the SDS-PAGE of the crude extract of Humulus pollens (Figure 6).

The Humulus pollen allergen could be identified in the serum of 10 mice. The molecular weight of serum proteins that had the maximum binding to allergen was ~25 kDa and
Subcutaneous immunotherapy for allergic asthma

Discussion

In this study, we evaluated the efficacy of subcutaneous immunotherapy of high-dose (100 μg) Humulus pollen allergen in a mouse model of allergic asthma induced by Humulus pollen. Penh is a commonly used dimensionless indicator for pulmonary function assessment, which is related to the mouse airway resistance and pressure in the pleura and can be measured by body plethysmography. Our results showed that high-dose and short-course immunotherapy could significantly improve the pulmonary function, alleviate airway inflammatory response and relieve allergic symptoms in asthmatic mice. What we’ve found is consistent with previous literatures [8, 9].

IgE is involved in the pathophysiological process of type I allergic reaction and plays an important role in the development of airway hyperresponsiveness in patients with asthma [10]. Serum tIgE level is positively correlated with the severity of asthma [11]. The serum tIgE level in the model group was significantly higher than that in the control group, which was consistent with previous studies [12, 13]. It has been reported that the expression of tIgE would either increase [14, 15] or remain steady after immunotherapy [16, 17]. In this study, however, we found that tIgE level in immunotherapy group was significantly elevated compared to that in model group, which was inconsistent with previous studies [16, 17]. IgE is mainly secreted by B cells. Akdis et al found that B cells showed no evidence of tolerance in the early stage of treatment [18]. Therefore, the elevated tIgE level after treatment might be related to shorter duration of immunotherapy. In addition, the concentration of allergen used in the present study was obviously higher than previous studies, which in another aspect could partially explain the why the expression of tIgE elevated following treatment. Serum sIgE expression remained steady after immunotherapy, which was also in accordance with previous studies [16, 17]. However, the kinetics of sIgE expression after immunotherapy is still uncertain. Some studies demonstrated that the level of sIgE reduced after immunotherapy [14, 19], whereas Van Ree et al suggested that sIgE might increase temporarily in the early stage of treatment and would gradually decline after months or years of treatment [20].

Figure 6. SDS-PAGE of Humulus pollen allergens. The amounts of Humulus protein sample on bands 1-3 were 30 μL (parallel hole), 20 μL (parallel hole) and 10 μL (parallel hole). The concentration was 1.5 μg/μL. The molecular weights of major proteins were ~80, ~70, ~38, ~30, ~26, ~15 and ~10 kDa.

Figure 7. Western blot analysis of Humulus pollen allergen that specifically binds to IgE. 1-10: 10 mice in the model group; P: Positive control; N: Negative control. The molecular weights of serum proteins that had the maximum binding to allergen were ~25 kDa (line 2, 3, 4, 6, 7, 8, 9) and ~38 kDa (line 2, 3, 4, 6, 8, 9). Other low (10-20 kDa), medium (25-35 kDa) and high (50-80 kDa) molecular weight proteins showed poor binding to the allergen (~38 kDa in 7 cases (70%) and 6 cases (60%), respectively. Other proteins with low (10-20 kDa), medium (25-35 kDa) or high (50-80 kDa) molecular weight showed poor binding to the allergen (Figure 7).
Subcutaneous immunotherapy for allergic asthma

were relieved regardless of the changes in IgE expression level. Yet no clear correlation between serum IgE levels and the remission of allergic clinical symptoms has been established. Changes in the serum IgE level could not explain the reduction of allergen response after immunotherapy.

The level of IgG1 and IgG2a represents the intensity of Th2 and Th1 immune response, respectively [21-26]. The increase of IgG2a level has been regarded as an indicator of effective immunotherapy. It has also been established that allergen could induce immune response to skew Th2-type to Th1-type in asthmatic mice [27-29]. In the present study, IgG2a level significantly increased after immunotherapy, which is consistent with previous study [30]; however, the increase in IgG1 following immunotherapy was inconsistent with previous studies [26, 31]. Bach et al [28] suggested that subcutaneous injection could induce Th2-type immune response. Jiménez-Saiz et al suggest that subcutaneous injection of allergens can cause minor lesions, inflammation and the recruitment of activated dendritic cells to draining lymph nodes, a process that induces the production of IgG1+ B cells in lymph node germinal centers and elevate serum allergen sIgG1 [32]. Other study has considered that the presence of non-protein components in crude extracts such as chitin, β-glucose can activate innate immune responses and promote inflammation through immunotherapy injection [33]. In this study, repeated subcutaneous injection of allergens was administrated, and the increase of sIgG1 was likely associated with repeated subcutaneous injection of allergens. Moreover, the immunotherapy process of daily subcutaneous injection might contribute to the induction of Th2-type immune response with elevated IgG1.

IL-5 and IL-13 are two major Th2 cytokines. IL-5 sustains the survival of eosinophil [34] and IL-13 stimulates the antibody class switching to IgE. As shown in the present study, the expression level of IL-5 and IL-13 was significantly higher in the model group than in the control group, suggesting that Th2-type immune response was induced in the model group, which was consistent with previous studies [17, 35]. It was reported that Th2 cytokines were significantly reduced after immunotherapy [27], while other studies showed that Th1 and Th2 cytokines did not change after immunotherapy [17]. In this study, the levels of Th1 cytokine IL-12 and Th2 cytokine IL-5/13 showed increasing tendencies after immunotherapy, although the differences were not statistically significant. It might be reasonable to conclude that both Th1 and Th2 immune responses could be enhanced after immunotherapy. However, this conclusion still merits further research with larger sample size.

Chinese researchers tested the immunoreactivity of Humulus pollen allergens in the human body and found that Humulus pollen contained more than 20 proteins. The molecular weight of major allergen ranged from 5 to 43 kDa. Proteins with molecular weight of 43-97.4 kDa had stronger immunogenicity but weaker antigenicity [36]. The immunoreactivity of allergens in mice has not yet been reported. Results of this study demonstrated that the molecular weight of main allergens of Humulus pollen-sensitized mice was ~25 kDa and ~38 kDa, which has provided a theoretical basis for the immunotherapy with allergenic components of Humulus allergens.

Despite our discovery, there are still some limitations in this study. The influence of subcutaneous injection on immunotherapy could not be determined because the control of PBS injection was not established in the immunotherapy. In addition, the main allergens of Humulus pollen that sensitized mice were not isolated and the efficacy of immunotherapy was not assessed using these allergens.

In conclusion, high-dose subcutaneous immunotherapy could effectively alleviate airway hyperresponsiveness and reduce airway inflammatory infiltration in allergic asthmatic mice.

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Disclosure of conflict of interest

None.

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Subcutaneous immunotherapy for allergic asthma


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