Bioscience, Biotechnology, and Biochemistry

ISSN: 0916-8451 (Print) 1347-6947 (Online) Journal homepage: http://www.tandfonline.com/loi/tbbb20

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To cite this article: Shiho Murakami, Sayuri Nakayama, Makoto Hattori & Tadashi Yoshida (2015) Establishment and characterization of a novel murine model for pollen allergy, Bioscience, Biotechnology, and Biochemistry, 79:9, 1447-1453, DOI: 10.1080/09168451.2015.1027654

To link to this article: http://dx.doi.org/10.1080/09168451.2015.1027654

Published online: 26 May 2015.

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Bioscience, Biotechnology, and Biochemistry, 2015
Vol. 79, No. 9, 1447–1453

Establishment and characterization of a novel murine model for pollen allergy

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Received January 21, 2015; accepted March 2, 2015
http://dx.doi.org/10.1080/09168451.2015.1027654

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Although there have been many studies revealing the mechanism and establishing the therapeutical method for allergic rhinitis, no suitable animal models for allergic rhinitis, especially for pollen allergy, are currently available. We therefore aimed in this study to develop a murine model producing IgE in response to an inhaled antigen without using any adjuvants. Ovalbumin (OVA)-specific T cell receptor transgenic mice (DO11.10) inhaled an OVA solution for one h, twice a week, for six weeks. The resulting increase of OVA-specific IgE in the serum was observed depending on the times of inhalation. Spleen cells from mice that had inhaled the antigen produced more IL-4 and less IFN-γ than those from the control mice *in vitro*. These results indicate that inhaled antigen enhanced the Th2-type responses and induced IgE production in a T cell-mediated manner. Our findings would contribute to studies on prevention and treatment of pollen allergy.

Key words: IgE; inhaled antigen; pollen allergy; TCR transgenic mice; Th2 response

There has recently been an increase in the number of allergic patients, especially in developed countries.[1–3] In particular, the increase in the number of patients suffering from pollen allergy is remarkable. It has been estimated that 40% of Japanese people have allergic rhinitis, including pollen allergy.[4] Although not a life-threatening disease, it does lead to a degradation in the quality of life and productivity of the patients having a substantially bad impact on the society.[5] It is therefore urgently necessary to reveal the mechanism involved in the onset of pollen allergy and to establish a therapeutical method for handling it.

Experiments using animal models are essential at a stage prior to clinical research in order to study various diseases. For example, ApoE knockout mice develop atherosclerosis,[6,7] and NC/Nga mice show skin lesions similar to those in human atopic dermatitis[8]; these mice have therefore been widely used in animal disease models for many studies. Among various allergic diseases, there have been many studies revealing the mechanism and establishing a therapeutical method for allergic rhinitis. However, no suitable animal models for allergic rhinitis, especially pollen allergy, are currently available. An artificial method had therefore been adopted as a model for pollen allergy in most previous studies,[9,10] whereby IgE production was induced in mice by i.p. injection of an antigen with some adjuvants and subsequent nasal administration of the antigen. Although clinical symptoms similar to those of pollen allergy can be observed with this model in response to intranasal administration of the antigen, the entire onset mechanism would be different from that in patients who had been sensitized by an inhaled antigen.

Many researchers have reported the results from experiments that applied such a model in evaluating the effect of anti-allergic foods. Sunada et al. have reported the effect of *Lactobacillus acidophilus* strain L-55 on experimental allergic rhinitis in BALB/c mice sensitized with OVA, alum, and the pertussis toxin.[11] They have shown that an oral administration of strain L-55 caused a significant decrease of antigen-specific IgE production in the serum and inhibited nasal rubbing and sneezing induced by an antigen challenge into the nasal cavity. Yatsuzuka et al. have also reported the anti-allergic properties of Usuhiratake (*Pleurotus pulmonarius*) by evaluating the inhibition of sneezing and nasal rubbing induced by the antigen in BALB/c mice that had been intraperitoneally sensitized with OVA, alum, and *B. pertussis*.[12] According to these reports, the effects of anti-allergic foods on such symptoms as sneezing could be evaluated using this model, although a difference in the induction mechanism of allergy might influence the mechanism causing the symptoms, resulting in a difference in the efficacy of anti-allergic foods on the symptoms. This model would therefore not be appropriate for evaluating the comprehensive effects of anti-allergic foods on pollen allergy, and it is necessary to use a more natural allergic model to evaluate anti-allergic foods.

A study has been reported which attempted to sensitize an animal only using an inhaled antigen without any adjuvant. Wilder et al. have reported that DO11.10, the same strain as that we used, developed airway hyperreactivity against an inhaled ovalbumin (OVA)

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Abbreviations: OVA, ovalmumin; TCR, T cell receptor.

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solution.[13] However, no apparent OVA-specific IgE in the serum was observed in their report, suggesting that their result was not based on the IgE-mediated type I allergy, which is typical in the patients with pollen allergy[5]. This is therefore also unsuitable for use as a pollen allergy model. Similar observations have also been reported by other groups.[14,15]

We aimed in this present study to develop a novel murine allergy model producing IgE in response to an inhaled antigen without using any adjuvant. We used DO11.10, a strain of OVA-specific T cell receptor (TCR) transgenic mice because this strain had already been used as a food allergy model.[16] These mice have been reported to experience a significant allergic reaction only by orally ingesting the antigen without using any mucosal adjuvant. We therefore expected to establish a more natural pollen allergic model using this strain. Not only the effects and working mechanism of anti-allergic foods, but also the mechanism involved in the onset of pollen allergy, such as the site of sensitization by the inhaled antigen, as well as the cell types and functions involved could be clarified using this model. This model would also be helpful for investigating the influence of such environmental factors as air pollutants on the onset of pollen allergy. Our findings are likely to contribute to studies on prevention and treatment of pollen allergy.

Materials and methods

Mice. DO11.10 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred in our university. T cells in the mice expressed TCR specific for OVA 323–339 bound with I-A^d molecules. All the mice that were used in the experiments were heterozygous (male and female, 6–16 weeks old) which were produced by breeding a male homozygote with female BALB/c mice. Female BALB/c mice (seven weeks old) were purchased from Clea Japan (Tokyo, Japan). All the mice were maintained and used in accordance with the guidelines for the care and use of experimental animals of Tokyo University of Agriculture and Technology.

Antigen sensitization. Sensitization to the inhaled antigen was induced by putting the mice in a box (22,000 cm^3) and letting them inhale 2 mg/mL of OVA (Sigma, St Louis, MO, USA)/saline or only saline for one h, twice a week, for six weeks using an ultrasonic humidifier (Ryohin Keikaku Co., Tokyo, Japan). The volume of OVA solution consumed during one-h treatment was approximately 20 mL. The mice were bled from the tail vein every two weeks. Sensitization to the oral antigen was induced by feeding the mice with a 20% OVA-containing diet (Oriental Yeast Co., Tokyo, Japan). The mice were bled from the tail vein every week or every two weeks.

Antibody production in the serum. Total and OVA-specific antibodies in the serum were measured by ELISA. Total IgE was measured using Maxisorp immunoplates (Nunc, Roskilde, Denmark) coated with purified R35-92 rat anti-mouse IgE mAb (BD Pharmingen, San Diego, CA, USA). Samples and standards were added after washing and blocking. Bound IgE was detected using R35-118 biotinylated rat anti-mouse IgE mAb (BD Pharmingen), and subsequently incubating with alkaline phosphatase streptavidin. An enzyme substrate (*p*-nitrophenol phosphate) was added, and the absorbance was determined at 405 nm. Maxisorp immunoplates coated with 0.01% OVA were used for measuring OVA-specific IgE, IgG1, and IgG2a. Samples and standards were added after washing and blocking. Bound antibodies were, respectively, detected using biotinylated rat anti-mouse IgE mAb, biotinylated rabbit anti-mouse IgG1 (Invitrogen, Carlsbad, California, USA) or biotinylated rabbit anti-mouse IgG2a (Invitrogen), and subsequently incubating with alkaline phosphatase streptavidin. An enzyme substrate (*p*-nitrophenol phosphate) was then added and the absorbance was determined at 405 nm. The results were shown as relative concentration to standard serum that had been obtained from other mice immunized with OVA.

Cell culture. Single-cell suspensions of splenocytes were prepared three days after the last inhalation of OVA. After depleting the erythrocytes, the spleen cell suspensions were cultured with or without OVA (100 μg/mL or 1000 μg/mL) in RPMI1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (Sigma), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 5×10^{-5} mol/L of 2-mercaptoethanol in 96-well plates (Corning, New York, USA). The supernatant of each well was collected after three days to determine the cytokine level.

Cytokine production in the culture supernatant. The supernatant of each well was collected at three days after antigen stimulation and measured by ELISA for cytokine production. To measure IL-4 and IFN-γ, Maxisorp immunoplates were coated with purified 11B11 rat anti-mouse IL-4 mAb (BD Pharmingen) or purified R4-6A2 rat anti-mouse IFN-γ (BD Pharmingen) antibody. Samples and standards were added after washing and blocking. Bound IL-4 and IFN-γ were, respectively, detected using BVD6-24G2 biotinylated rat anti-mouse IL-4 (BD Pharmingen), or XMG1.2 biotinylated rat anti-mouse IFN-γ (BD Pharmingen), before incubating with alkaline phosphatase streptavidin. The enzyme substrate (*p*-nitrophenol phosphate) was added, and the absorbance was determined at 405 nm.

Histological analysis. Heads were obtained from the DO11.10 mice three days after the last inhalation of OVA or saline and fixed in 10% neutral buffered formalin. Paraffin-embedded sections at three different positions of the nose were prepared and stained with hematoxylin and eosin (Sapporo General Pathology Laboratory, Sapporo, Japan). The appearance of eosinophilic bodies was also evaluated by Sapporo General Pathology Laboratory.

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Statistical analysis. The statistical analysis was performed by Student's *t*-test or the Tukey–Kramer method. The analysis by Tukey–Kramer method was carried out among samples obtained at the same time point, and different letters shown in the figures indicate significant difference. A p value of less than 0.05 was considered significant.

Results

Induction of antigen-specific IgE in DO11.10 mice was apparently accomplished by inhalation of the OVA solution

DO11.10 mice were made to inhale OVA by means of an ultrasonic humidifier in an attempt to induce serum IgE in response to the inhaled antigen. The increase of OVA-specific IgE in the serum was observed depending on the times of inhalation (Fig. 1(A)). In contrast, this kind of IgE induction was not apparent in DO11.10 mice that had inhaled saline, indicating that we had successfully established a novel allergic model for pollen allergy, which could be induced without any help from an adjuvant. Although BALB/c mice have the same genetic background as DO11.10, they did not produce IgE, even after inhaling OVA. These results indicate that the inhalation of OVA induced IgE production in an antigen-specific manner in DO11.10 mice via a T cell-dependent mechanism.

We also examined the serum level of other classes of OVA-specific antibodies. DO11.10 mice which had inhaled OVA produced higher levels of IgG1 and IgG2a antibodies than did BALB/c mice (Fig. 1(B) and (C)).

Inhalation of OVA enhanced IL-4 production, while inhibiting IFN-γ from spleen cells of the mice

To confirm that the inhaled antigen induced IgE production was mediated by antigen-specific T cell response, this T cell response was analyzed by culturing splenocytes after the inhalation of OVA for six weeks. The IL-4 produced by spleen cells from the mice that had inhaled the OVA solution was higher than that in the control mice, while the IFN-γ production was inhibited as a result of the OVA inhalation (Fig. 2(A) and (B)), suggesting that the Th2 predominant response induced by the inhalation of OVA was involved in the IgE production.

Similar levels of antigen-specific IgE were induced in the food allergy and inhaled allergy models

We next compared the degree of elevated IgE level in the novel murine allergy model to that in a food allergic model which had been well characterized in the previous studies. The level of antigen-specific IgE in the pollen allergy model mice was comparable with that in the food allergy model mice (Fig. 3(A)). In contrast, the total amount of IgE produced in food allergy model mice was significantly higher than that in the pollen allergy model mice (Fig. 3(B)). Additionally, IgE production in the food allergy model reached a peak one or two weeks after starting the 20% OVA diet

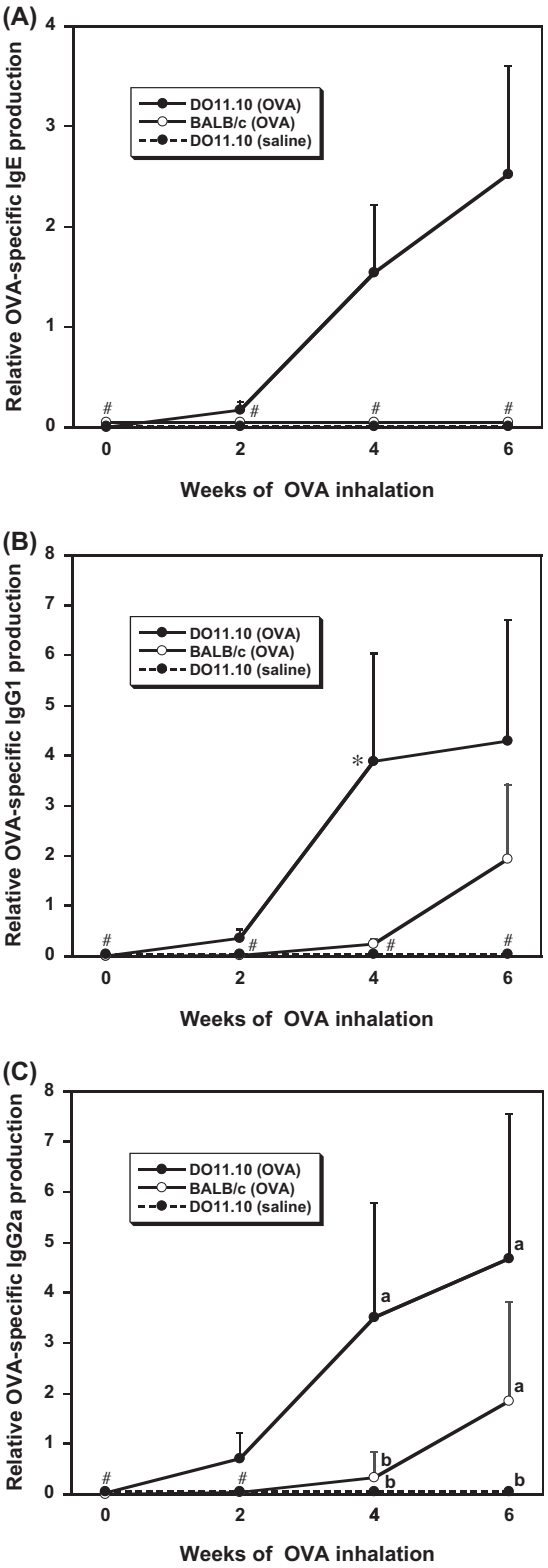

Fig. 1. Effect of OVA inhalation on antigen-specific antibody production in DO11.10 mice.

Notes: DO11.10 mice inhaled 2 mg/mL of OVA/saline (filled circles, solid line; $n = 5$) or saline (filled circles, dotted line; $n = 3$) for one h, twice a week, for six weeks by means of an ultrasonic humidifier. BALB/c mice also inhaled 2 mg/mL of OVA/saline (unfilled circles, solid line; $n = 4$) in the same manner. The level of OVA-specific IgE, IgG1, and IgG2a in the serum was measured by ELISA. The results are representative of two independent experiments. Data are presented as the mean value ± SD. #Not detected. *Significantly different between two different strains at $p < 0.05$.

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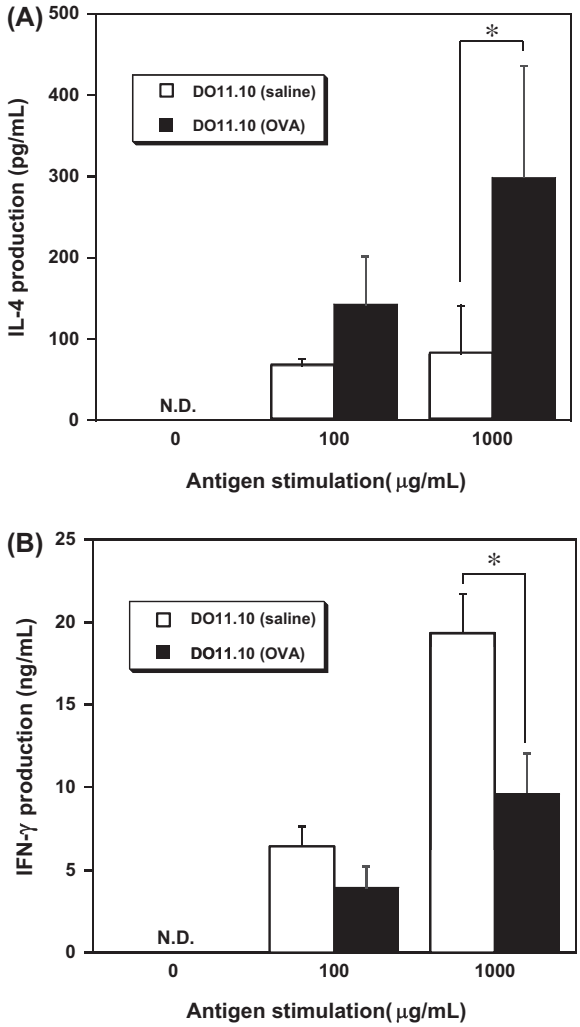

Fig. 2. Effect of OVA inhalation on cytokine production by spleen cells from DO11.10 mice.

Notes: Spleen cell suspensions were prepared three days after the last inhalation of OVA (filled bars; $n = 5$) or saline (unfilled bars; $n = 3$). The spleen cells were cultured for three days and (A) IL-4 production and (B) IFN-γ production were measured by ELISA. The results are representative of four independent experiments. Data are presented as the mean value ± SD. *Significantly different from the control group at $p < 0.05$. N.D.: not detected.

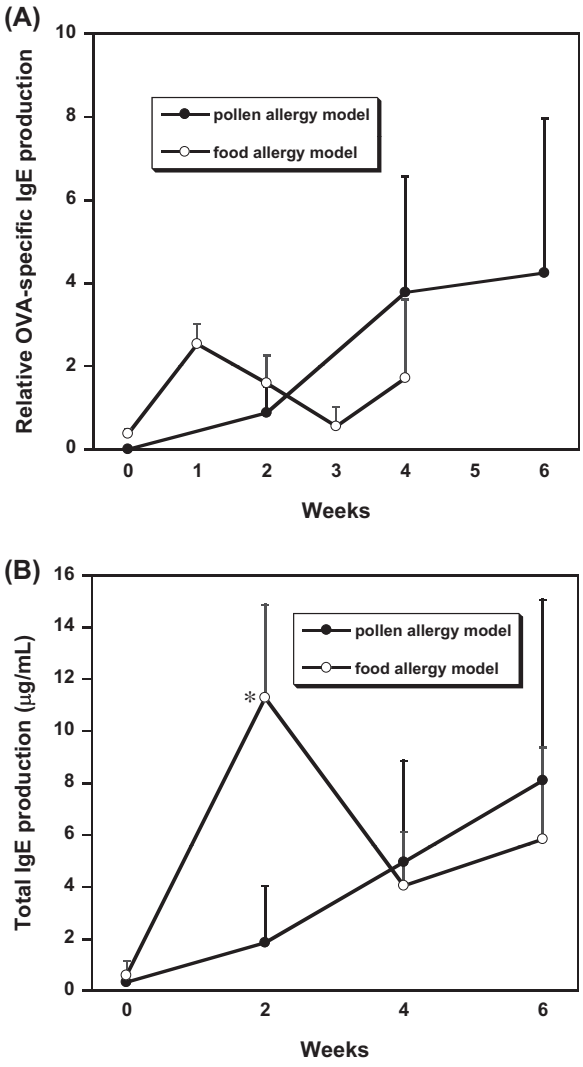

Fig. 3. Concentration of OVA-specific IgE antibodies and total IgE antibodies in the sera from the pollen allergy model mice and food allergy model mice.

Notes: The pollen allergy model mice (filled circle, solid line; $n = 5$) inhaled OVA for six weeks, and the food allergy model mice (unfilled circle, solid line; $n = 4$) were fed a 20% OVA diet for six weeks. The OVA-specific IgE (A) and total IgE (B) concentrations in the sera were each measured by ELISA. The results are representative of two independent experiments. Data were presented as the mean value ± SD. *Significantly different between two groups at $p < 0.05$.

for antigen-specific or total IgE, respectively, and then gradually decreased. However, the inhaled allergy model did not show this trend at least until week 6.

Many eosinophilic bodies appeared in the nasal mucosa after inhalation of the antigen

We then assessed if any allergic symptoms were apparent in these mice. However, no such clinical symptoms as sneezing or increased nasal secretion were apparent either immediately after each inhalation or at the time between each inhalation. We next observed any histological changes in the nasal mucosa of the model mice and found the appearance of many eosinophilic bodies only in the mice which had inhaled OVA (Fig. 4). On the other hand, we did not observe the invasion of any lymphocytes or mast cells. Fig. 4(B) shows a picture at the nasal position of level 1, and similar results were apparent at three different nasal positions (Fig. 4(A) and Table 1).

Discussion

The aim of this study was to establish a pollen allergy model that would be suitable for various investigations. We developed a novel murine model which produced IgE in response to an inhaled antigen without using any adjuvants. We could examine the induction mechanism for pollen allergy and develop anti-allergic foods and drugs using this allergy model.

The use of DO11.10 OVA-specific TCR transgenic mice was crucial to establishing the stable pollen allergy model. Previous studies have reported many pollen allergy models.[13, 17–21] Some of these studies could induce allergy without using any adjuvants.[20,21] Renz et al. reported that IgE production was induced in

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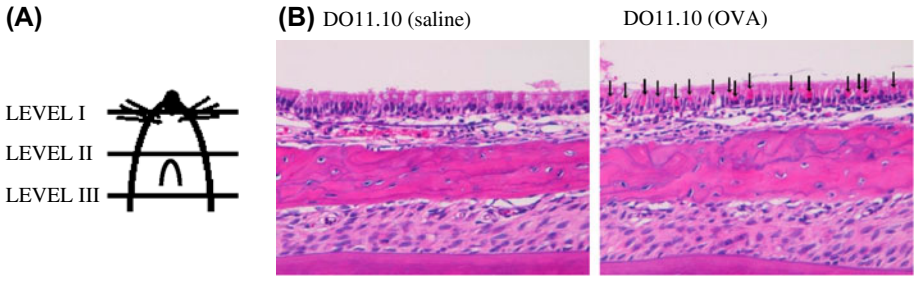

Fig. 4. Histological changes in the mucous layer of the nose (×280).

Notes: (A) The position where the histological sections were obtained. (B) DO11.10 mice inhaled OVA ($n = 4$) or saline ($n = 3$) for six weeks. Nasal sections were obtained three days after the last inhalation from the position at level I and stained by hematoxylin and eosin. Eosinophilic bodies are indicated by arrows.

Table 1. Appearance of eosinophilic bodies at three different positions in the nose.

Group	DO11.10 (saline)									DO11.10 (OVA)											
Mouse No.	1			2			3			4			5			6			7		
Level	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Eosinophilic bodies in the nasal mucosa	±	−	−	−	±	−	−	−	−	+	+	−	+	+	−	±	+	+	++	++	+

Notes: −: negative, ±: minimal, +: mild, ++: moderate.

Nasal sections were obtained from DO11.10 mice ($n = 3$ for the control and $n = 4$ for the pollen allergy) as described in Fig. 4. The appearance of eosinophilic bodies at three different positions of the nose was evaluated. The pictures of mouse Nos. 2 and 4 at level 1 are shown in Fig. 4.

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the serum of BALB/c mice that had inhaled an aerosolized OVA solution without using any adjuvants.[20] Although this result had been reported, most of the other studies used an artificial experimental method by which IgE was induced by i.p. immunization of the antigen with an alum adjuvant and then subsequent antigen challenges to the nose to obtain a strong and stable allergic status.[11,12] We did not find any apparent IgE induction in our present study when BALB/c mice were treated with the OVA solution, the reason for their different result from ours being unknown. Considering that the IgE level was expressed as units in their report, a small change in IgE concentration that we could not detect might have occurred in their study. In fact, the histological change observed in our model was not apparent in either the nasal mucosa or pulmonary mucous membrane in their study. This difference might indicate that the level of response against the inhaled antigen was too weak to cause a histological change in wild-type mice. Since BALB/c mice possess a variety of T cells recognizing various antigens, the number of T cells which recognize OVA might be too small to induce strong and stable IgE response. Barrett et al. used DO11.10 in their study.[21] They reported that the exposure of aerosolized OVA to DO11.10 induced IgE production similar with our results. However, the level of OVA-specific IgE was comparable to that of BALB/c mice and that induced by irrelevant antigen, cigarette smoke. This result suggests that the level of OVA-specific IgE observed in the study was also quite low because the level of IgE induced by inhaled antigen was very weak in BALB/c as we discussed before. The reason why the level of OVA-specific IgE was similar between DO11.10 and BALB/c is not sure, but it is considered that the amount of inhaled antigen was too high to obtain high level of OVA-specific IgE. We actually observed that higher concentration of antigen induced lower level of IgE (data not shown). The higher concentration of antigen might induce nasal tolerance, resulting in lower level of IgE production. Similar phenomenon was also observed in food allergy model mice because of the induction of oral tolerance (Fig. 3(A)).

We did not observed any IgE production in BALB/c mice, while the apparent induction of OVA-specific IgG1 and IgG2a in the serum of BALB/c mice after inhaling the antigen was observed, even though the level was less than that induced in DO11.10 mice, suggesting that inhalation of the antigen could induce an immune response in BALB/c mice, although a stronger immune response was needed to induce strong and stable IgE induction. It is also suggested that the intensity of IgE induced in DO11.10 mice was stronger than that in wild-type animals, while the induction mechanism would be similar with the naturally occurring pollen allergy. Therefore, we can apply this transgenic model for examining the onset mechanism of pollen allergy and development of anti-allergic agents.

We observed that spleen cells from mice that had inhaled the antigen produced more IL-4 and less IFN-γ than those from the control mice in response to *in vitro* antigen stimulation. IL-4 is known to be secreted by Th2 cells and to enhance the production of IgG1 and IgE by activating B cells. In contrast, it is known that IFN-γ is secreted by Th1 cells and suppresses the production of IgE with the activation of cellular immunity.[22,23] It is suggested that the Th1/Th2 balance was skewed toward Th2 dominance in the mice that had inhaled OVA. This result shows that inhaling the antigen enhanced the Th2-type response antigen-specifically and induced IgE production in a T cell-mediated manner with our experimental system. This agrees with an earlier report that allergic patients presented Th2-dominant immune reactions.[24] The reason why Th2

skewing occurred in this study is not clear, but we speculate as follows: first, the T cell responsiveness tended to be skewed toward Th2 when sensitized naturally by an antigen without using any adjuvant,[25] particularly in DO11.10 mice, because BALB/c mice, the genetic background strain for DO11.10 mice, are recognized as a Th2-dominant strain. Second, it could also be considered that intranasal tolerance might have been induced. It has been reported that the administration of an antigen into nasal mucosa induced T cell tolerance,[26,27] and our previous study has shown that Th1 cells were rendered tolerant much more easily than Th2 cells,[28] suggesting that inhaling a small amount of the antigen only induced tolerance for Th1 cells and not for Th2 cells.

In many other studies with mice, sensitizing the antigen with some adjuvants before its nasal administration has been adopted to observe the allergic reaction in response to a nasal antigen.[17–19] However, the sensitizing mechanism occurring in the model would be different from that in patients with pollen allergy who had been sensitized by inhaling the antigen. That model would therefore be inappropriate for analyzing the mechanism involved in the onset of pollen allergy and investigating the effect and mechanism of anti-allergic foods or medicines for pollen allergy. Since a difference in the induction mechanism would greatly influence the effect of such agents, understanding the mechanism involved in the onset of pollen allergy, such as the site of sensitization and the cell types concerned, would be important for developing them. As already mentioned, the IgE response in our model would be induced by the same mechanism as that in patients with pollen allergy, indicating the validity of our model for doing such research. It has been reported about the induction mechanism of pollen allergy that IgE class switching occurred on the nasal mucosa of allergic patients but not healthy controls.[29] However, it has not been clarified if the IgE class switching requires preceded sensitization at another site. We in this study showed that cytokine production of splenocytes was affected by inhaled antigen. The migration to nasal mucosa of cells that had been sensitized at spleen or other lymphoid tissues might be important. In addition, we can compare the effect of an anti-allergic agent using two different allergy models established from the same strain of mice: pollen allergy and food allergy.

It has been suggested that pollens have adjuvant activity as well as antigenicity,[30] although this has not been clearly determined. We anticipate that such adjuvant activity of pollens could be evaluated using DO11.10 mice to inhale an OVA solution containing pollens according to our methodology. Furthermore, the influence of some environmental substances which are believed to worsen allergic symptoms such as PM2.5[31,32] could be also evaluated.

We did not observe any clinical symptoms such as nasal secretion and sneezing throughout the experimental period, although a substantial histological change indicating an inflammatory reaction in the mucous layer of the nose was found. It is difficult to analyze this kind of change in a patient, so our model would be useful to examine the histological reactions induced by pollen allergy in detail. We can also evaluate the effect of anti-allergic foods and drugs based on the degree of inflammation as well as the IgE level. We also consider that sneezing and nasal secretion could be observed by inhaling more antigen with mice that had already inhaled the antigen several times because the level of antigen-specific IgE induced with the pollen allergy model was comparable to that with the food allergy model, in which a strong skin anaphylaxis reaction has been reported by intracutaneous injection of an antigen.[16]

We found that the amount of antigen-specific IgE in the serum of pollen allergy model mice was comparable to that of food allergy model mice. Since mice sensitized by inhaling the antigen would be exposed to a lower amount of the antigen than the food allergy model mice, we deduced that IgE produced by the pollen allergy model mice would be lower. Contrary to our expectations, a relatively large amount of IgE was in fact induced in the mice. This level of IgE induction would be enough as a pollen allergy model. In contrast, no IgE production was apparent in the study by Wilder et al., even though they used the same strain of mice as ours.[13] It is considered that the period of inhalation was insufficient to induce IgE production in their study. We also analyzed total IgE production in both allergy model mice. Interestingly, the total IgE produced by the food allergy model mice was significantly higher than that by the pollen allergy model mice. This might indicate that a large amount of oral antigen strongly stimulated the immune system and induced antibody production in both an antigen-specific manner as well as by a bystander effect, suggesting that food allergy would be a critical trigger on the allergic March.[33]

The concentration of OVA-specific IgE peaked one - week after starting oral administration of the antigen and then gradually diminished in the food allergy model. However, these phenomena were not apparent in the pollen allergy model mice. These results show that oral tolerance might have been induced in the food allergy model mice by ingesting a large amount of the antigen, while nasal tolerance was not induced in the pollen allergy model at least for Th2 cells. It seems that the quantity of antigen inhaled was insufficient for inducing tolerance in the pollen allergy model mice, although it was sufficient to induce significant IgE production. We indeed observed that nasal tolerance was induced at the Th2 level even in the pollen allergy model mice when they had inhaled 10 mg/mL of the OVA solution (data not shown).

The pollen allergy model established in this study proved useful for clarifying the mechanism involved at the onset of pollen allergy. Our findings would also greatly contribute to studies on the prevention and treatment of allergies. The effect of medicines or anti-allergic foods might vary depending on the kind of allergy, although no studies that had examined such issues have been carried out. Examining the different effects of foods and medicines on allergic responses using various allergic models, including this novel pollen allergy model, would provide novel insight.

Disclosure statement

No potential conflict of interest was reported by the authors.

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