

A new murine model of allergic rhinitis by repeated intranasal Cry j 1 challenge

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ABSTRACT

To evaluate the long-lasting effects of new therapeutic approaches to allergies, we established a new model of allergic rhinitis by repeated challenges with intranasal Cry j 1, a common Japanese cedar (*Cryptomeria japonica*) pollen allergen, in B10.S mice. We sensitized B10.S mice subcutaneously with Cry j 1/alum three times at 1-week intervals. Five weeks after the final sensitization, we challenged the mice by instilling Cry j 1 intranasally for 5 consecutive days starting 1 day after intranasal histamine pretreatment (challenge-1). We challenged the mice by instilling histamine and Cry j 1 intranasally again 12 weeks later (challenge-2). There were significantly more sneezes after challenge-2 than challenge-1. Cry j 1-specific IgE levels in serum were significantly increased in both challenge-1 and 2 after continuous nasal antigen challenge. Serum levels of anti-Cry j 1 IgE in challenge-2 was 2.3 times higher than after challenge-1. Thus, we have established a new model of seasonal allergic rhinitis in B10.S mice by repeated intranasal antigen challenge, and this model may help elucidate mechanisms of allergic rhinitis and the development of new drugs.

Japanese cedar (*Cryptomeria japonica*, or Cj) pollinosis has reached a prevalence of 15% of the Japanese population (4). Patients with Cj pollinosis have symptoms such as sneezing, watery rhinorrhea, and nasal blockage. Therapeutic options include allergen avoidance, immunotherapy, and pharmacotherapy, particularly H1-antihistamines and topical corticosteroids (19). Allergen immunotherapy involves subcutaneous injections of gradually increasing quantities of specific allergens, such as venom, pollens, cat dander, dust mites, or modified allergens, to an allergic patient. Immunotherapy is an effective treatment for allergic rhinitis (11).

Multiple animal models of allergic rhinitis have

been used to evaluate pharmacotherapy and immunotherapy (6, 9, 10, 13, 14). Nasal blockage and sneezing occur in guinea pigs given repeated inhalation challenges with Cj pollen (10). Antigen-induced nasal symptoms, late-phase nasal eosinophilia, and serum levels of antigen-specific IgE antibody were evaluated in an ovalbumin-sensitized BALB/c mouse model of allergic rhinitis (9, 13). We also previously reported on an allergic rhinitis model in B10.S mice (19). These can be valuable models for analyzing the pathogenesis of the disease and developing new therapeutic drugs, but can not evaluate long-term symptoms or efficacy of new therapeutic approaches.

To study the relevant immunological mechanisms of Cj pollinosis and establish new therapeutic approaches, we established a model of Cj pollinosis that mimics the major features of human allergic rhinitis in B10.S mice challenged intranasally with histamine and Cry j 1. Cry j 1 is one of the major allergens in Japanese cedar pollen, which is basic

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glycoprotein with a molecular weight of 41-45,000 (16).

MATERIALS AND METHODS

Animals. Female B10.S mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The animals were housed under controlled temperature ($21 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 15\%$), fed a standard diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan), and given water *ad libitum*. All experiments were performed according to the "Guiding Principles for the Care and Use of Laboratory Animals" approved by The Japanese Pharmacological Society.

Antigen. Cry j 1 was purified from Japanese cedar pollen as described previously (3). The absence of Cry j 2 in the purified Cry j 1 preparation was confirmed by Western blot analysis with two monoclonal antibodies (anti-Cry j 1 and anti-Cry j 2; Hayashibara Biochemical Laboratories Inc., Okayama, Japan). Cry j 1 was 88% pure as determined by densitometric scanning after sodium dodecyl sulfate polyacrylamide gel electrophoresis and coomassie brilliant blue staining.

Sensitization and nasal antigen challenge. Mice were sensitized with 10 μg of Cry j 1 mixed with 4 mg of aluminum hydroxide gel (Imject Alum; Pearce, Rockford, IL, USA) by subcutaneous injection once a week for three consecutive weeks. Five weeks after the final sensitization of Cry j 1 and prior to the intranasal Cry j 1 challenge, 1 $\mu\text{g}/\text{mL}$ of histamine dihydrochloride (Nacalai Tesque Inc., Kyoto, Japan) in phosphate buffered saline (PBS) was dropped into both nostrils (10 μL per nostril) on the day before the first intranasal antigen challenge. Following histamine pretreatment, 10 μL of Cry j 1 (200 $\mu\text{g}/\text{mL}$ in PBS) was dropped into each nostril using a micropipette, for five consecutive days. The intranasal Cry j 1 challenge was performed twice (challenge-1 and challenge-2), with challenge-2 performed 12 weeks after challenge-1. Soon after the last intranasal antigen challenge, the number of sneezes was counted for 5 min.

Blood was collected via the tail vein to evaluate serum levels of Cry j 1-specific IgE (Fig. 1). Cry j 1-specific IgG1 and IgG2a antibodies were also measured in serum two weeks after challenge-2.

Evaluation of serum levels of Cry j 1-specific IgE. The day after the final intranasal antigen challenge, the Cry j 1-specific IgE titer in the serum was evaluated by an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates (F16 Black Maxi-sorp; Nalge Nunc International) were coated with 100 μL of purified rat anti-mouse IgE monoclonal antibody (50 $\mu\text{g}/\text{mL}$; BD Bioscience, San Jose, CA, USA) in bicarbonate buffer (0.1 mol/L, pH 8.2) overnight at 4°C . After removal of the coating solution, the plates were washed and blocked with PBS containing 1% bovine serum albumin for 30 min at room temperature (RT). After blocking, serum samples and standards were added to the plate in duplicate. Serum standards were obtained by serial dilution after intraperitoneal immunization of a mouse with Cry j 1 and arbitrarily assigned a value of 10,000 relative units (R.U.) /mL of Cry j 1-specific IgE. Plates were then washed and incubated with 100 μL of 1/2000 diluted Cedar Pollen Allergen Cry j 1-Biotin (Hayashibara Biochemical Laboratories Inc.) for 1 h at RT. The plate was then washed and subsequently incubated with streptavidin- β -galactosidase conjugate (Molecular Probes, Eugene, OR, USA) for 30 min at RT. After a final wash, 100 μL of substrate solution, consisting of Tris-HCl with 0.034 mg/mL of 4-methylumbelliferin- β -D-galactopyranoside, was added to the well for 2 h at 37°C . The reaction was stopped with 100 μL of glycine-NaOH, 0.2 mol/L, pH 10.3, and the fluorescence intensity was measured by a microplate fluorescence reader (Fluoroskan Ascent; Thermo Electron Corp., Waltham, MA, USA) at 355 nm (excitation) and 460 nm (emission).

Evaluation of serum levels of Cry j 1-specific IgG1 and IgG2a. Ninety-six-well plates were coated with 50 μL of Cry j 1 (10 $\mu\text{g}/\text{mL}$) overnight at RT and the plates were blocked with PBS containing 1% BSA. Then the plates were washed and incubated

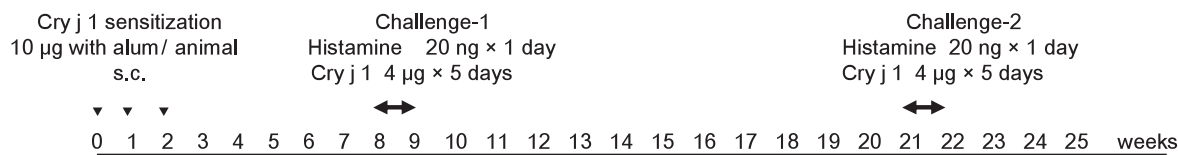


Fig. 1 Experimental protocols

with serum samples for 2 h at RT. After incubation, peroxidase-labeled anti-mouse IgG1 or IgG2a was added to the plate for 1 h at RT. Tetramethylbenzidine substrate solution (TMB Substrate Kit; Pearce, Rockford, IL, USA) was added to the plate after washing. Finally, 4 mol/L of phosphoric acid was added to stop the reaction and the absorbance at 450 nm was measured with a microplate reader.

RESULTS

Sneeze response

We measured the number of sneezes after five days continuous antigen challenge. The number of sneezes after challenge-1 and challenge-2 was 13.4 ± 3.8 and 50.9 ± 7.9 , respectively (Fig. 2). The number of sneezes after challenge-2 was 3.8 times higher than challenge-1 ($p < 0.01$).

Serum anti-Cry j 1 IgE antibody

Challenge-1 significantly increased serum levels of anti-Cry j 1 IgE, from 452 ± 97 R.U./mL to 7050 ± 1179 R.U./mL (Fig. 3). Serum levels of anti-Cry j 1 IgE three weeks after challenge-1 were 15465 ± 2988 R.U./mL, and gradually decreased thereafter before challenge-2. Challenge-2 significantly increased IgE serum levels from 5830 ± 1178 R.U./mL to 15978 ± 2835 R.U./mL, peaking at two weeks after challenge-2 (35500 ± 5411 R.U./mL), and decreasing thereafter. Changes in IgE levels during

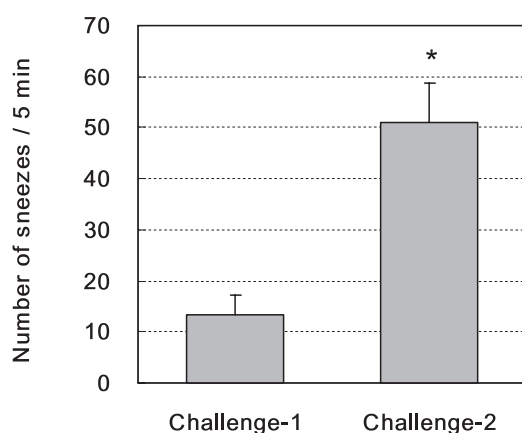


Fig. 2 Number of sneezes after challenge-1 and challenge-2. Five weeks after the last subcutaneous Cry j 1 sensitization, 10 μ L of histamine was applied intranasally, and on the next day, Cry j 1 was dropped into both nostrils for 5 days (challenge-1). Five weeks after the challenge-1, nasal histamine and antigen challenge was given again (challenge-2). Each value represents the mean $\pm$ S.E. of 24 mice. *: $p < 0.05$ (Aspin-Welch test), significantly different from challenge-1.

challenge-1 (6597 ± 1162 R.U./mL) were lower than challenge-2 (10148 ± 2643 R.U./mL).

Serum anti-Cry j 1 IgG1 and IgG2a antibody

Serum levels of anti-Cry j 1 IgG1 and IgG2a antibodies were measured two weeks after challenge-2. IgG1 levels increased after nasal antigen challenge, whereas IgG2a did not (Fig. 4).

DISCUSSION

Many allergic rhinitis models mimic the major features of allergic rhinitis in humans (6, 9, 10, 13, 14). To study the relevant immunological mechanisms of Cj pollinosis and establish new therapeutic approaches, we had previously developed an allergic rhinitis model in B10.S mice elicited by histamine pretreatment and continuous intranasal antigen challenges for 5 days (17). This model of allergic rhinitis produces sneezing, eosinophil infiltration into the nasal mucosa, and Cry j 1-specific IgE production, and should prove useful in studying human cedar pollinosis. We also examined the efficacy of an immunotherapeutic peptide in this model (7, 17). However, this model and others only mimic short-term rhinitis and not seasonal rhinitis. Repeated exposure to pollen increases patient sensitivity and their condition gets worse (3). Here, we developed a model with strong, prolonged symptoms with repeated intranasal Cry j 1 challenges separated by a 3-month interval in B10.S mice.

Our sneeze data showed that repeated intranasal antigen challenge enhanced sensitivity to the allergen at the nasal mucosa, as it does in humans. In humans, pollen-specific IgE increases during pollen season, but decreases without further antigen exposure (8). In our model, anti-Cry j 1 IgE increased

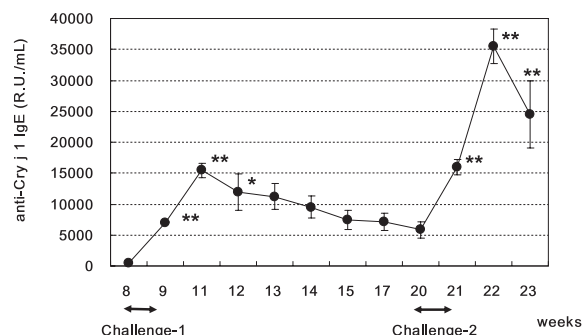


Fig. 3 Anti-Cry j 1 IgE antibody in serum measured by ELISA. Each value represents the mean $\pm$ S.E. of 24 mice. *: $p < 0.05$, **: $p < 0.01$ (Aspin-Welch test), significantly different from before the challenge-1(8W).

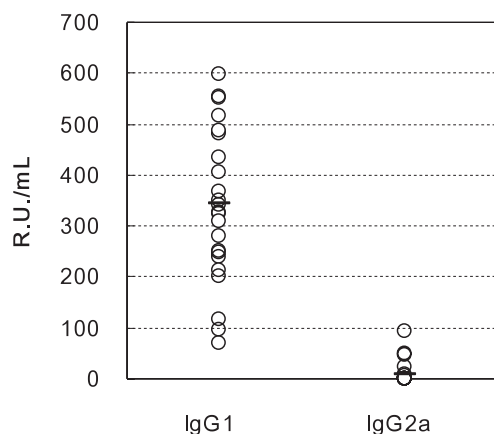


Fig. 4 Anti-Cry j 1 IgG1 and IgG2a antibody in serum after two weeks of the challenge-2 measured by ELISA. Each dot represents the individual value and “—” shows the mean value of 24 mice.

after the challenge-1, decreased gradually to a steady state, and then increased to greater levels after challenge-2. These changes in serum IgE levels are similar in patients with seasonal rhinitis.

In mice, IgG1 and IgG2a levels are associated with Th2 and Th1 responses, respectively (1, 12), prompting us to measure serum levels of antigen-specific IgG1 and IgG2a. In this model, IgG1 levels increased after nasal antigen challenge, whereas IgG2a did not, suggesting that our model is characterized by Th2-dominated inflammation, which also is true in humans. IgG2a production is stimulated by IFN- γ and IgG1 production is stimulated by IL-4. These two IgG isotypes reflect levels of IFN- γ and IL-2, which are Th1 cytokines, and IL-4, IL-5 and IL-6, which are Th2 cytokines (5). In humans, IgE reflects the Th2 response, whereas IgG4 reflects the Th1 response. Allergen-specific IgE and IgG4 levels reflect immunotherapy efficacy (2, 15). The concomitant increase in IgG2a levels and decline in IgG1 points to a shift from a Th2 response to a Th1-dominated response. Measuring these antigen-specific antibodies reveals therapeutic mechanisms.

This model mimics the major features of allergic rhinitis in humans, and shows satisfactory symptomatic and pathophysiological changes in allergic rhinitis. IFN- γ and IL-4 are important cytokines in the pathogenesis of allergic diseases. Further analysis of cytokines in this model may lead to new therapeutic strategies that target induction of Th2/Th1 in allergic rhinitis. Thus, we succeeded in developing a model of seasonal allergic rhinitis in B10.S mice by repeated intranasal antigen challenge.

REFERENCES

1. Aberer W, Hawranek T, Reider N, Schuster C, Sturm G and Kränke B (2007) Immunoglobulin E and G antibody profiles to grass pollen allergens during a short course of sublingual immunotherapy. *J Investig Allergol Clin Immunol* **17**, 131–136.
2. Chakraborty P, Roy I, Chatterjee S, Chanda S and Gupta-Bharracharya S (2006) Phoenix sylvestris Roxb pollen allergy: a 2-year randomized controlled trial and follow-up study of immunotherapy in patients with seasonal allergy in an agricultural area of West Bengal, India. *J Investig Allergol Clin Immunol* **16**, 377–384.
3. Ehnthage A, Kölbeck KG, Mossberg B and Juto JE (2002) Nasal and bronchial histamine responsiveness in pollen-exposed patients with seasonal rhinitis. *ORL J Otorhinolaryngol Relat Spec* **64**, 191–199.
4. Kaneko Y, Motohashi Y, Nakamura H, Endo T and Eboshida A (2005) Increasing prevalence of Japanese cedar pollinosis: a meta-regression analysis. *Int Arch Allergy Immunol* **136**, 365–371.
5. Kim DK, Joo KH and Chung MS (2007) Changes of cytokine mRNA expression and IgG responses in rats infected with *Capillaria hepatica*. *Korean J Parasitol* **45**, 95–102.
6. Kim JH, Mun YJ, Ahn SH, Park JS and Woo WH (2001) Induction of oral tolerance to Japanese cedar pollen. *Arch Pharm Res* **24**, 557–563.
7. Kozutsumi D, Tsunematsu M, Yamaji T, Murakami R, Yokoyama M and Kino K (2006) Cry-consensus peptide, a novel peptide for immunotherapy of Japanese cedar pollinosis, induces Th1-predominant response in Cry j 1-sensitized B10.S mice. *Biol Pharm Bull* **29**, 2506–2509.
8. Magnusson J, Lin XP, Dahlman-Höglund A, Hanson L LA, Teleme E, Magnusson O, Bengtsson U and Ahlstedt S (2003) Seasonal intestinal inflammation in patients with birch pollen allergy. *J Allergy Clin Immunol* **112**, 45–50.
9. Murasugi T, Nakagami Y, Yoshitomi T, Hirahara K, Yamashita M, Taniguchi Y, Sakaguchi M and Ito K (2005) Oral administration of a T cell epitope inhibits symptoms and reactions of allergic rhinitis in Japanese cedar pollen allergen-sensitized mice. *Eur J Pharmacol* **510**, 143–148.
10. Nabe T, Mizutani N, Shimizu K, Takenaka H and Kohno S (1998) Development of pollen-induced allergic rhinitis with early and late phase nasal blockage in guinea pigs. *Inflamm Res* **47**, 369–374.
11. Nelson HS (1999) Mechanisms of intranasal steroids in the management of upper respiratory allergic diseases. *Allergy Clin Immunol* **104**, S138–143.
12. Nouri-Aria KT, Wachholz PA, Francis JN, Jacobson MR, Walker SM, Wilcock LK, Staple SQ, Aalberse RC, Till SJ and Durham SR (2004) Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol* **172**, 3252–3259.
13. Sato J, Asakura K, Murakami M, Ueda T and Katakura A (1999) Topical CTLA4-Ig suppresses ongoing mucosal immune response in presensitized murine model of allergic rhinitis. *Int Arch Allergy Immunol* **119**, 197–204.
14. Takagi H, Hiroi T, Yang L, Tada Y, Yuki Y, Takamura K, Ishimitsu R, Kawauchi H, Kiyono H and Takaiwa F (2005) A rice-based edible vaccine expressing multiple T cell epitopes induces oral tolerance for inhibition of Th2-mediated IgE responses. *Proc Natl Acad Sci USA* **102**, 17525–17530.
15. Till SJ, Francis JN, Nouri-Aria K and Durham SR (2004) Mechanisms of immunotherapy. *J Allergy Clin Immunol* **113**,

- 1025–1034.
16. Toda M, Sato H, Takebe Y, Taniguchi Y, Saito S, Inouye S, Takemori T and Sakaguchi M (2000) Inhibition of immunoglobulin E response to Japanese cedar pollen allergen (Cry j 1) in mice by DNA immunization: different outcomes dependent on the plasmid DNA inoculation method. *Immunology* **99**, 179–86.
 17. Tsunematsu M, Yamaji T, Kozutsumi D, Murakami R, Kimura S and Kino K (2007) Effect of Cry-consensus peptide, a novel recombinant peptide for immunotherapy of Japanese cedar pollinosis, on an experimental allergic rhinitis model in B10.S mice. *Allergol Int* **56**, 465–472.
 18. Tsunematsu M, Yamaji T, Kozutsumi D, Murakami R, Kimura S and Kino K (2007) Establishment of an allergic rhinitis model in mice for the evaluation of nasal symptoms. *Life Sci* **80**, 1388–1394.
 19. Wang DY (2002) Treatment of allergic rhinitis: H1-antihistamines and intranasal steroids. *Curr Drug Targets Inflamm Allergy* **1**, 215–220.

