

Cryptomeria japonica-Induced Allergic Conjunctivitis in Mice

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Japanese cedar pollen (*Cryptomeria japonica*, Cry j) is the most common allergen causing pollinosis in Japan. However, short ragweed pollen is used commonly as the antigen for experimentally-induced allergic conjunctivitis (EC) and Cry j-induced EC in mice has not been published. We actively immunized BALB/c mice with Cry j, and then performed a challenge with eye drops containing Cry j. We evaluated the early phase and late phase reactions in the conjunctiva, using Evans blue dye leakage and eosinophil infiltration, respectively. Significant inhibition of the early phase reaction was observed following pre-challenge with eye drops that block histamine H1 receptor in the conjunctiva. Thus, Cry j-induced EC appears to represent a suitable model for the study of pollinosis in Japan.

Key words allergic conjunctivitis; *cryptomeria japonica*; histamine H1 receptor blocker; early phase reaction; late phase reaction

Japanese cedar pollen (*Cryptomeria japonica*, Cry j)-induced pollinosis is the most widespread allergic disease in Japan, affecting over 15% of the population.¹⁾ Allergic conjunctivitis (AC) is one of the most uncomfortable clinical symptoms of pollinosis.²⁾ It is mediated by an interaction between mast cells and antigen (Ag)-specific IgE and is treated with eye drops containing medications that stabilize mast cells and block histamine H1 receptors.³⁾ However, the effectiveness of these treatments can be unsatisfactory and clinics often administer steroids, which cause complications such as glaucoma.⁴⁾

Animal models play an important role in our search to understand the mechanisms underlying AC and in the identification of more effective therapeutics. In animal models of AC, short ragweed pollen (RW) is most common antigen used.^{5–10)} However, Cry j-induced AC is far more common in Japan than RW-induced AC.¹¹⁾ Since different allergens may induce different immune response characteristics,¹²⁾ investigations using Cry j-induced conjunctivitis can provide information that is relevant to Japanese pollinosis patients. Although there have been 3 reports available with regard to Cry j-induced conjunctivitis in guinea pigs,^{13–15)} there have been no reports with regard to Cry j-induced conjunctivitis in mice. Here, we aimed to establish Cry j-induced experimental conjunctivitis (EC) in mice and to evaluate whether or not this model is suitable for the evaluation of eye drops containing anti-allergic agents.

MATERIALS AND METHODS

Mice BALB/c mice were purchased from Charles River Laboratories, Yokohama, Japan or from Japan SLC, Hamamatsu, Japan. These mice were maintained under pathogen-free conditions at the animal facility of Santen Nara Research and Development Center and Kochi Medical School. Male mice were immunized at 5-weeks-old. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The number of mice in each experiment is at least more than 5.

Reagents Cry j was purchased from Hayashibara, Japan.

Aluminum hydroxide (alum) was prepared with 42 mg/ml NaOH and 659 mg/ml Al₂(SO₄)₃. Evan's blue (EB) was purchased from Sigma (St. Louis, MO, U.S.A.). Histamine H1 receptor-blocking eye drops (levocabastine hydrochloride ophthalmic solution, olopatadin hydrochloride ophthalmic solution and ketotifen fumarate ophthalmic solution) were obtained from Santen Pharmaceutical, Alcon JAPAN and Novartis Pharma, respectively.

Preparation of a Cry j/Alum Emulsion and Induction of EC by Active Immunization Cry j was suspended in phosphate buffered saline (PBS) and allowed to stand at 0 °C. Different doses of Cry j were adsorbed on alum (2.5 mg) and injected intraperitoneally twice, on the day of immunization (day 0) and at day 5. On day 11, the right eyes of the mice were challenged with Cry j in PBS (1.2 mg per 2 µl). The control groups were the mice that were immunized but not challenged (no challenge group) or the mice that were not immunized but challenged (no sensitization group).

ELISA Detection of Total IgE in Serum IgE antibodies were assayed using a mouse IgE ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan). In brief, microtiter plates were coated with anti-mouse IgE mAb and diluted serum from either naive or immunized mice (1 mg of Cry j) was added to the wells for overnight incubation at 4 °C. Plates were washed and enzyme-linked goat anti-mouse IgE Ab was added to the wells. Following incubation for 2 h at room temperature, plates were washed again and then incubated with TMB solution for 30 min at room temperature. The enzyme reaction was stopped with 1 N sulfuric acid and plates were read at 450 nm.

Evaluation of the Early Phase Reaction (EPR) Using Extravasation of EB EB (15 µg/g body weight) was injected intravenously into Cry j-immunized mice, immediately prior to challenge with eye drops containing Cry j suspended in PBS. After 30 min, mice were sacrificed and their conjunctivas were harvested. EB was extracted for 48 h in 2 ml of a 0.5% Na₂SO₄ and acetone mixture (3:7). After centrifugation, the absorbance (620 nm) of the supernatant was determined using a spectrophotometer. Data are presented as EB content/weight of conjunctiva.

Evaluation of the Late Phase Reaction (LPR) Using

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Conjunctival Eosinophil Infiltration Eyes (including the conjunctivas) were harvested 24 h after the Cry j challenge and fixed in 10% buffered formalin. Vertical 2 μ m-thick sections were cut and stained with Giemsa. Infiltrating eosinophils present in the lamina propria mucosae of the tarsal and bulbar conjunctivas were counted in the each section by two observers given blinded samples. Sections counted were chosen from the central portion of the eye, which included the pupil and optic nerve head. Data are presented as means $\pm$ S.E.M. for all mice examined.

Effects of Histamine H1 Receptor-Blocking Eye Drops on EPR Three types of eye drop, which contained histamine H1 receptor-blocking medication, were applied to the conjunctiva (2 μ l per eye) 30 min prior to a Cry j challenge. Immediately before the challenge, EB was injected intravenously as described above. The challenge was performed using eye drops containing Cry j suspended in PBS and after 30 min, EB extravasation was evaluated as described above. Percentage inhibition was determined as $\{1 - (\text{leakage in histamine H1 receptor blocker-treated group} - \text{leakage of no challenge group}) / (\text{leakage of saline-treated group} - \text{leakage of no challenge group})\} \times 100$.

Statistical Analyses Analyses were performed using the Student's *t*-test or Dunnett's multiple comparison test. Probability values (*p*) < 0.05 were considered significant.

RESULTS AND DISCUSSION

Early Phase Reaction (EPR) in Cry j-Induced EC BALB/c mice were injected intraperitoneally on day 0 and day 5, with different doses of Cry j (0.2, 0.4 or 1 mg per injection) emulsified in alum. On day 11, EB was injected intravenously immediately prior to challenge with eye drops containing Cry j in the right eyes. After 30 min, the conjunctivas were harvested and the EB content was determined. Following Cry j challenge, the conjunctival EB contents of Cry j-immunized animals increased significantly compared with those of the controls (Fig. 1A). In addition, the EB contents increased in an Ag dose-dependent manner (Fig. 1A). Cry j-immunized mice also exhibited significantly higher total serum IgE levels than naive mice (Fig. 1B). Thus, we have confirmed that an EPR can be induced by immunization and challenge with Cry j.

Late Phase Reaction (LPR) in Cry j-Induced EC Next, we investigated whether or not a LPR could be induced in Cry j-induced EC. Eosinophil infiltration into the conjunctiva is considered to be a marker for LPR in the conjunctiva, since the number of infiltrating eosinophils into the conjunctiva increases as the severity of AC increases.¹⁶⁾ Therefore, we counted the numbers of conjunctival eosinophils to evaluate LPR in the conjunctiva. Mice were immunized and challenged as above, without EB treatment. Twenty-four hours after the challenge, conjunctivas were harvested to determine conjunctival eosinophil numbers. The Cry j challenge induced conjunctival eosinophil infiltration in animals sensitized to Cry j, whereas no infiltration was observed in the control animals (no challenge group or no sensitization group, Fig. 2). Thus, LPR was induced in Cry j-induced EC.

EPR Is Inhibited by Eye Drops Containing Histamine H1 Receptor Blockers Finally, to investigate whether or not Cry j-induced EC is suitable for evaluating the efficacy of

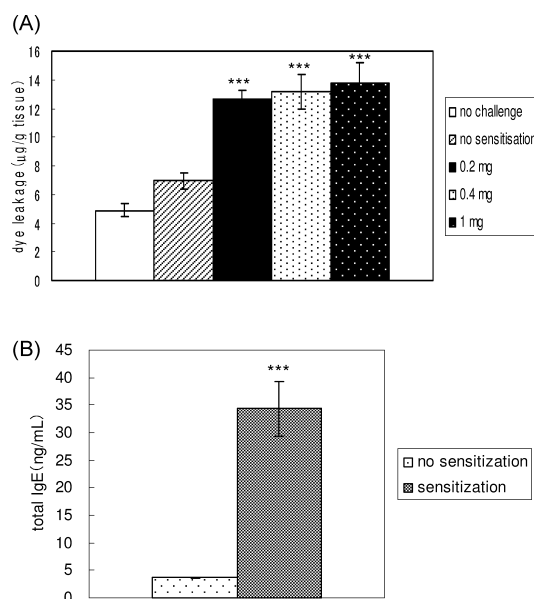


Fig. 1. EPR in Cry j-Induced EC

BALB/c mice were actively immunized with different doses of Cry j in alum. After 11 d, the mice were challenged with eye drops containing Cry j. (A) Immediately prior to the Cry j challenge, Evan's blue (EB) was injected intravenously. After 30 min, the conjunctivas were harvested and their EB contents were determined. As controls, we used mice that were immunized but not challenged with Cry j (no challenge group) or not immunized but challenged with Cry j (no sensitization group). Data are expressed as EB content/weight of conjunctiva. ****p* < 0.001 vs. no challenge group, Dunnett's multiple comparison test. (B) On day 11, the Cry j-immunized mice (1 mg of Cry j) were sacrificed for blood collection. Sera were prepared and total serum IgE levels were determined by ELISA. ****p* < 0.001 vs. no sensitization group, Student's *t*-test.

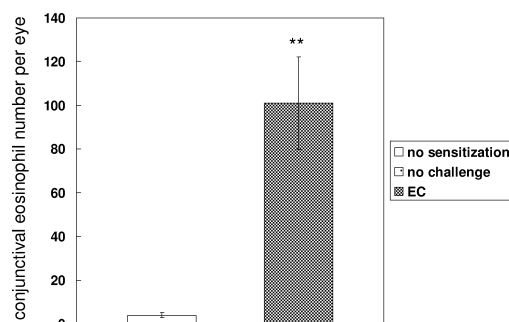


Fig. 2. LPR in Cry j-Induced EC

BALB/c mice were actively immunized with 0.2 mg Cry j in alum. After 11 d, the mice were challenged with eye drops containing Cry j. Conjunctivas were harvested after 24 h, in order to determine conjunctival eosinophil infiltration. Data are presented as conjunctival eosinophil numbers per eye. ***p* < 0.01 vs. no sensitization or no challenge group, Dunnett's multiple comparison test.

anti-allergic eye drops, we investigated 3 types of commercially-available eye drops containing blockers for the histamine H1 receptor. Eye drops were administered 30 min prior to Ag challenge of actively immunized mice. The Ag challenge was administered and after a further 30 min, EB was injected intravenously. Treatment with eye drops containing histamine H1 receptor blockers resulted in significant inhibition of EB extravasation (Fig. 3). Thus, Cry j-induced EC appears to be a suitable model for the evaluation of therapeutic eye drops.

CONCLUSION

Similar to data obtained for RW-induced EC,⁵⁻¹⁰⁾ we observed both EPR and LPR in Cry j-induced EC. Further-

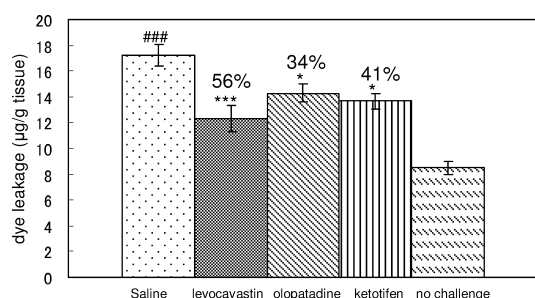


Fig. 3. Effects of Anti-histamine H1 Receptor-Blocking Eye Drops on the EPR

BABL/c mice were actively immunized with 0.2 mg of Cry j in alum. After 11 d, the mice were challenged with eye drops containing Cry j. Thirty minutes prior to the challenge, 3 types of anti-histamine H1 receptor-blocking eye drops were applied to the right eyes and EB was injected intravenously immediately before the challenge. Conjunctivas were harvested 30 min after the challenge conjunctival EB contents were determined. Data are expressed as EB content/weight of conjunctiva. Percentage inhibition is indicated. * $p < 0.05$ vs. saline group, Dunnett's multiple comparison test. *** $p < 0.001$ vs. saline group, Dunnett's multiple comparison test. ### $p < 0.001$ vs. no challenge group, Student's t -test.

more, we observed clear inhibition of EPR following treatment with eye drops containing histamine H1 receptor blockers. Taken together, our results indicate that Cry j-induced EC is a suitable model for human AC, in which Cry j is the target Ag.

We have investigated the roles Th2 cells play in the development of RW-induced EC. From the data in a previous report,¹⁰ it was confirmed that Ag-specific Th2 cells, but not Th1 cells, induced eosinophil infiltration into the conjunctiva. Moreover, over-expression of suppressor of cytokine signaling (SOCS)3 in T cells deteriorated EC, while that of SOCS5 reduced the severity of EC.¹⁰ As shown in Fig. 1B, IgE levels in serum were significantly higher in mice developing Cry j-induced EC than the control mice. This data suggests that Th2-mediated response plays a role in the development of Cry j-induced EC, similar to RW-induced EC. Together with the data that cyclosporin A-containing eye drops significantly suppressed the development of RW-induced EC,¹⁷ negative regulation of Th2-mediated response in the conjunctiva such by dominant-negative SOCS3 would be a therapeutic strategy in patients with AC induced by Cry j.

Recently, Cry j-induced allergic conjunctivitis in guinea pigs has been reported.^{13–15} More recently, the clinical fea-

tures of pollinosis such as sneezing and nasal rubbing were reported for Cry j2 immunization followed by a Cry j2 intranasal challenge.¹⁸ However, Cry j-induced conjunctivitis in mice has not been reported. Because of availability of monoclonal antibodies and gene-knockout animals, it would be advantageous to use Cry j-induced conjunctivitis in mice for detailed analysis and therapeutic studies. Thus, we expect that the present study will help elucidate the mechanisms underlying Cry j-induced AC and that this animal will prove useful for investigation of the effects of anti-pollinosis drugs and in particular those that target conjunctivitis.

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