

Experimental Allergic Conjunctivitis in Guinea Pigs Induced by Japanese Cedar Pollen

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We report a new experimental allergic conjunctivitis with Japanese cedar pollen as antigen in guinea pigs, and the immunological characteristics of this model were also elucidated. Allergic conjunctivitis was developed by immunization in guinea pigs with a mixture containing Japanese cedar pollen and killed *Bordetella pertussis*. When local application of Japanese cedar pollen suspension 14 d after systemic immunization was performed every 3 d, remarkable conjunctivitis was observed from 20 to 35 d. Increase in vascular permeability and decrease in histamine contents of the conjunctiva were also observed after local application of antigen. Passive cutaneous anaphylactic (PCA) reactions revealed that both IgG- and IgE-rich antibodies were produced in this model. Chlorpheniramine, ketotifen and levocabastine were effective in inhibiting cedar pollen-induced conjunctivitis. Although a high concentration was needed, tranilast and amlexanox also showed significant inhibition of conjunctivitis induced by cedar pollen.

Key words allergic conjunctivitis; Japanese cedar pollen; IgG antibody; chlorpheniramine; ketotifen; amlexanox

We have reported that experimental models for allergic conjunctivitis were developed by topical applications of histamine in non-sensitized guinea pigs and of antigen in sensitized guinea pigs.¹⁾ In these models it was found that histamine plays an important role in the process leading to allergic inflammation. That is, topical application of antigen resulted in a release of histamine from the conjunctiva, and lacrimal histamine contents were increased after antigen challenge in sensitized guinea pigs.²⁾ On the other hand, it is well known that allergic conjunctivitis in humans is a common symptom in IgE-mediated allergic diseases, and major sources eliciting these understood diseases are pollens from grasses, house dust mites, molds and Japanese cedar (*Cryptomeria japonica*).³⁾ Twenty years ago, allergic conjunctivitis induced by Japanese cedar pollen was rare, but the incidence of this disease is now on the increase, caused by abundant and widespread air pollution in Japan. However, literature dealing with the animal model for allergic conjunctivitis induced by Japanese cedar pollen is scanty.⁴⁾ It is well recognized that an immediate hypersensitivity reaction in allergic conjunctivitis caused by Japanese cedar pollen in humans depends on IgE antibodies, but guinea pigs are reported to have 2 types of homocytotropic antibodies, IgG₁ and IgE antibodies.⁵⁾

In this study, we describe a new method for producing experimental allergic conjunctivitis with Japanese cedar pollen in guinea pigs and the immunological characteristics of this model. The effects of certain antiallergic drugs on the model are also elucidated.

MATERIALS AND METHODS

Animals Male guinea pigs (200–300 g, Nippon SLC, Shizuoka, Japan) were used. The animals were housed in a temperature-controlled room at 24±2 °C with 55±15% humidity and were given food and water *ad libitum*. At least 5 animals were used in each group.

Reagents The following reagents were obtained from the sources shown in parentheses: Japanese cedar pollen (Torii Pharmaceutical, Tokyo, Japan), *Bordetella pertussis* in

active microorganism suspension (Kitasato Institute Research Center for Biology, Saitama, Japan), histamine dihydrochloride (Nacalai Tesque, Kyoto, Japan), Evans blue (Wako, Osaka, Japan), perchloric acid (Wako), affinity purified anti-guinea pig IgG-F(c) [goat] (Rockland Inc., Gilbertsville, PA, U.S.A.), chlorpheniramine hydrochloride (Wako), ketotifen fumarate (Sankyo, Tokyo, Japan), levocabastine hydrochloride (ophthalmic solution, Janssen-Kyowa, Tokyo, Japan), tranilast (Kissei, Nagano, Japan) and amlexanox (Elics®, ophthalmic solution, Senju, Osaka, Japan). All other reagents used were of the highest quality commercially available.

Sensitization and Challenge Forty animals were used in the present study. The animals were immunized by subcutaneous injection in the back of 1 ml of a mixture containing 20 mg cedar pollen and 10¹⁰ killed *Bordetella pertussis*. Seven days later, a booster immunization of the same mixture was given. After this second sensitization, the animals were repeatedly challenged/boosted by drops in the eyes of 10 µl of the cedar pollen suspension (300 µg/µl) every 3 d from 14 to 53 d.

Antigen-Induced Conjunctivitis Conjunctivitis was induced by application of 10 µl cedar pollen suspension (300 µg/µl) into the eyes, and severity of inflammation was estimated as follows: 0=no symptoms; 1=slight hyperemia; 2=severe hyperemia; 3=severe hyperemia and slight edema; and 4=severe edema.

Histamine Determination in the Conjunctiva Twenty-three days after the first immunization, histamine content in the conjunctiva was measured. Thirty minutes after 10 µl antigen application (cedar pollen suspension 300 µg/µl), guinea pig conjunctiva were carefully excised, weighed and washed twice with saline. The tissues were homogenized with 0.4 N perchloric acid and placed in an ice bath for 1 h. After centrifugation at 400×g, for 20 min at 4 °C, histamine content in the supernatant was determined by an automated fluorometric assay.⁶⁾

Changes in Conjunctival Vascular Permeability Twenty-six days after the first immunization, changes in conjunctival vascular permeability were measured. Five minutes

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after 10 μ l antigen application (cedar pollen suspension, 300 μ g/ μ l), 1% Evans blue solution (0.25 ml/100 g) was injected intravenously. Thirty minutes thereafter, the conjunctiva was removed, and Evans blue was extracted from the material with 0.5 ml of 1 N KOH solution for 12 h at 37 °C, and 4.5 ml of H₃PO₄-acetone (0.6 N H₃PO₄: acetone=5:13) was added and mixed well. After centrifugation at 400 $\times$ *g* for 20 min, the amount of extracted dye was determined using a spectrophotometer (Model U-2000, Hitachi, Tokyo, Japan) at 620 nm.

Preparation of Japanese Cedar Pollen Extracts Cedar pollen extracts were prepared according to the method of Ishizaki.⁷⁾ Briefly, 20 ml of 0.125 M NH₄HCO₃ solution was added to 1 g of cedar pollen, and extracted with stirring for 24 h at 4 °C. The solution was then centrifuged at 10000 $\times$ *g* for 30 min, and the supernatant was used as sample.

Passive Cutaneous Anaphylaxis (PCA) Reaction for IgE and IgG The IgE and IgG titers in the serum were determined by PCA reaction according to the method of Ovary *et al.*⁸⁾ Blood specimens were obtained from the hind legs. The doubling dilutions of serum obtained from sensitized guinea pigs were injected intradermally in volumes of 0.1 ml into the shaved backs of normal guinea pigs. After 4 h or 7 d, each animal was given an intravenous injection of 1 ml of physiological saline containing cedar pollen extracts (1 mg protein/ml) and 1% Evans blue (0.25 ml/100 g body weight). Thirty minutes thereafter, the guinea pigs were sacrificed, the dorsal back skin was peeled off, and the diameter of the blue spot on the underside of the skin was measured.

PCA Reaction for Affinity Purified Anti-guinea Pig IgG-Fc (IgG-Fc) To study whether or not PCA reaction induced by the serum obtained in the present study contained IgG antibody, the reaction was examined by the method described above using guinea pig serum absorbed with IgG-Fc. Guinea pig sera (1:32 dilution) were absorbed with IgG-Fc (2.0 mg/ml) at 1:20, 1:50, 1:100, 1:200 and 1:500 dilutions at room temperature for 2 h.

Drug Effects Twenty-nine, 32 and 35 d after the first immunization, 10 μ l of cedar pollen suspension (300 μ g/ μ l) was applied to the eyes 15 min after drug administration.

Statistical Analysis Values were expressed as mean $\pm$ S.E.M. The Mann-Whitney U test or ANOVA with Dunnett's test was used to calculate the statistical difference between the means of the test and control groups. A probability value of less than 0.05 was considered significant.

RESULTS

Conjunctivitis Induced by Cedar Pollen Changes in the severity of conjunctivitis induced by cedar pollen suspension are shown in Fig. 1a. When local application of cedar pollen suspension (300 μ g/ μ l) beginning 14 d after systemic immunization was performed every 3 d, remarkable conjunctivitis was observed from 20 to 35 d. On the 20th day, effects of some concentrations of cedar pollen suspension were examined; suspension understood without repeating caused no conjunctivitis at a concentration of 30 μ g/ μ l, but at concentrations of 100 and 300 μ g/ μ l, a dose-related severity of inflammation was observed. No conjunctivitis was observed when 300 μ g/ μ l of cedar pollen was applied to the eyes in unsensitized animals (data not shown) (Fig. 1b).

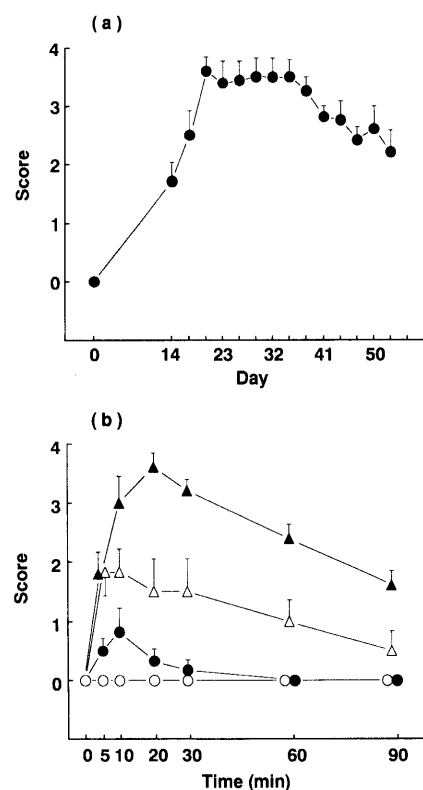


Fig. 1. Changes in the Severity of Cedar Pollen-Induced Conjunctivitis in Guinea Pigs

(a) Time-course study. Each value shows mean $\pm$ S.E.M. (*n*=40). 300 μ g/ μ l of cedar pollen suspension was applied locally. (b) Dose-response study. Each value shows mean $\pm$ S.E.M. (*n*=5). $\circ$, control; $\bullet$, 30 μ g/ μ l; $\triangle$, 100 μ g/ μ l; $\blacktriangle$, 300 μ g/ μ l.

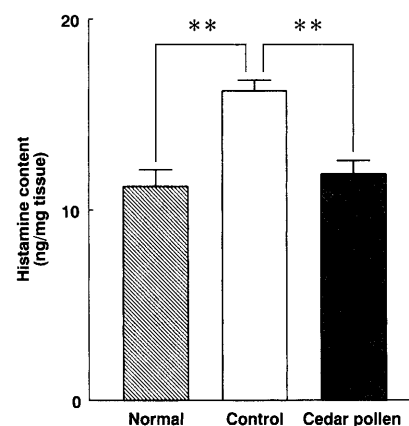


Fig. 2. Effect of Cedar Pollen on Conjunctival Histamine Content in Guinea Pigs

Histamine content in the conjunctiva was measured 30 min after antigen application. Each value shows mean $\pm$ S.E.M. (*n*=5). Normal, non-sensitized animals; control, sensitized animals, saline; cedar pollen, sensitized animals, cedar pollen. **: Significantly different from control at *p*<0.01.

Changes in Histamine Content Histamine content in the conjunctiva was measured to learn whether cedar pollen suspension (300 μ g/ μ l) causes histamine release from mast cells in the conjunctiva of guinea pigs. As shown in Fig. 2, the histamine content was increased significantly after cedar pollen sensitization compared with before sensitization, while it was decreased significantly after application of cedar pollen suspension.

Change in Vascular Permeability As shown in Fig. 3, dye content in the conjunctiva was also significantly in-

creased by application of the suspension ($300 \mu\text{g}/\mu\text{l}$), suggesting that vascular permeability was increased by this application.

The 4-h and 7-d PCA Reactions during the Chal-

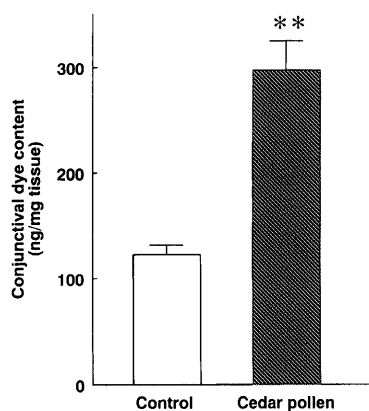


Fig. 3. Effect of Cedar Pollen on Conjunctival Dye Content in Guinea Pigs

Conjunctival dye content was measured 30 min after antigen application. Each value shows mean $\pm$ S.E.M. ($n=5$). Control, sensitized animals, saline; cedar pollen, sensitized animals, cedar pollen. **: Significantly different from control at $p < 0.01$.

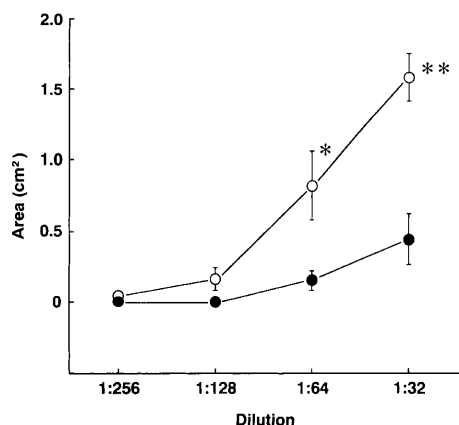


Fig. 4. PCA Reaction Induced by Anti-cedar Pollen Antibody in Guinea Pigs

Each value shows mean $\pm$ S.E.M. ($n=5$). $\circ$, 4 h; $\bullet$, 7 d. *, **: Significantly different from 7-d sensitized group at $p < 0.01$ and $p < 0.05$, respectively.

lenges/Boosters of Pollen Instillation On the 20th day after the 1st immunization, sera were obtained to estimate IgG and IgE antibody reactions using 4-h and 7-d PCAs, respectively. As shown in Fig. 4, a significant effect was observed in 4-h PCA with a titer of 1:64 and 1:32 dilutions. Seven-days PCA was also elevated with a titer of 1:32 dilution. The 4-h PCA reaction was not abolished with heat (56°C , 4 h) (Fig. 5a), but the PCA reaction was significantly abolished by antibody absorbed with IgG-Fc antibody at concentrations of 40 and 100 $\mu\text{g}/\text{ml}$ (Fig. 5b).

Effect of Anti-allergic Drugs Twenty-nine, 32 and 35 d

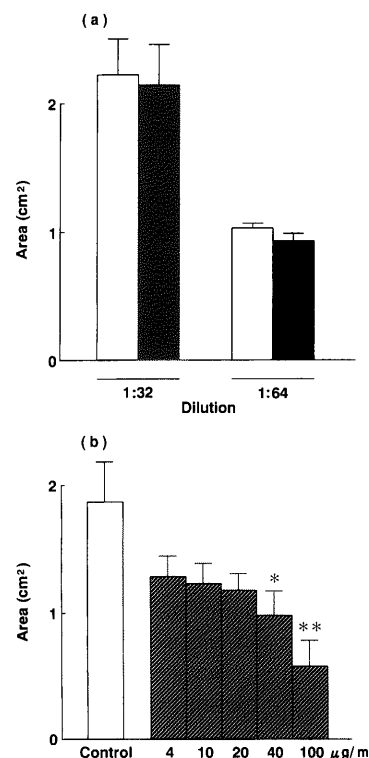


Fig. 5. PCA Reaction Induced by Antibody Treatment by Heat and Absorbing with IgG-Fc in Guinea Pigs

(a) Effect of incubation at 56°C for 4 h. Each value shows mean $\pm$ S.E.M. ($n=5$). $\square$, control; $\blacksquare$, incubation at 56°C for 4 h. (b) Effect of antibody absorbed with IgG-Fc. Each value shows mean $\pm$ S.E.M. ($n=5$). *, **: Significantly different from control at $p < 0.01$ and $p < 0.05$, respectively.

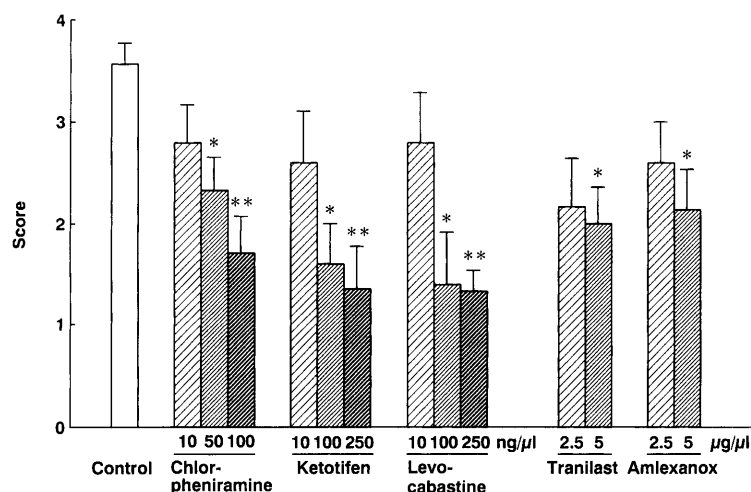


Fig. 6. Effects of Certain Antiallergic Drugs on Cedar Pollen-Induced Conjunctivitis in Guinea Pigs

Each value shows mean $\pm$ S.E.M. ($n=6$). *, **: Significantly different from the control at $p < 0.01$ and $p < 0.05$, respectively.

after immunization, 10 μ l of cedar pollen suspension (300 μ g/ μ l) was applied to the eyes 15 min after drug administration. Chlorpheniramine showed a dose-related effect on cedar pollen-induced conjunctivitis, and a significant effect was observed at 50 and 100 ng/ μ l. Both ketotifen and levocabastine also caused inhibition of this conjunctivitis. A significant effect of both drugs was observed at 100 and 250 ng/ μ l. Although a relatively high concentration was needed, tranilast (5 μ g/ μ l) and amlexanox (5 μ g/ μ l) also showed significant inhibition of conjunctivitis induced by cedar pollen.

DISCUSSION

In the present study, we succeeded in developing a model for allergic conjunctivitis using Japanese cedar pollen in guinea pigs. We have reported that antigen-induced conjunctivitis was provoked by use of egg albumin as antigen.¹⁾ The difference between allergic conjunctivitis induced by Japanese cedar pollen and that induced by egg albumin is as follows. The extent of clinical inflammation of allergic conjunctivitis induced by the two substances was almost the same. However, in the conjunctivitis induced by Japanese cedar pollen, the latency to peak effect of clinical symptoms was long, and the duration was also long compared with the conjunctivitis induced by egg albumin.¹⁾ Histamine content in the conjunctiva was also increased by sensitization to the pollen, and instillation of the pollen led to a decrease in histamine content of the conjunctiva, indicating that histamine was released by the antigen challenge. Almost the same results were obtained with allergic conjunctivitis sensitized with egg albumin in guinea pigs.¹⁾ Ishizaki⁷⁾ has reported that the number of mast cells in the conjunctiva was increased by sensitization with cedar pollen, and after challenge with the pollen these counts were decreased. These findings correspond with the view that histamine plays an important role in allergic conjunctivitis induced by Japanese cedar pollen. An increase in the vascular permeability of the conjunctiva induced by the pollen was also observed to the same extent as in that induced by egg albumin.

It was found in the present study that both 4-h and 7-d PCAs showed a positive reaction. Four-hours PCA reaction was higher than that of 7-d PCA reaction, and the 4-h PCA reaction was not abolished with heat (56 °C, 4 h). PCA reaction induced by IgG antibodies may be elicited from 15 min to 6–8 h after intradermal sensitization, while reaction induced by IgE antibodies may be elicited after 10 d of sensitization.⁹⁾ Furthermore, IgE antibodies were reported to be inactivated by heating at 56 °C for 30 min to 4 h.¹⁰⁾ It is generally accepted that guinea pigs have 2 types of homocytotropic antibodies, IgG₁ and IgE antibodies.¹¹⁾ As shown above, 4-h PCA reaction was significantly inhibited by using the antiserum absorbed with IgG-Fc antibody. Therefore, it is reasonable to presume that antiserum obtained in the present study was IgG-rich antibody.

As shown here, chlorpheniramine, ketotifen and levo-

cabastine were effective in inhibiting cedar pollen-induced conjunctivitis in guinea pigs. We previously showed that in allergic conjunctivitis induced by egg albumin, histamine plays an important role in the process leading to allergic inflammation.¹⁾ As shown in the present data, the effects of these 3 drugs were almost the same. However, it is well recognized that ketotifen shows more potent H₁-antagonistic activity than chlorpheniramine and levocabastine on the contraction of guinea pig ileum induced by histamine.¹²⁾ These differences can be accounted for by the fact that some putative chemical mediators also participate in cedar pollen-induced conjunctivitis.¹³⁾ Although a high concentration was needed, tranilast and amlexanox significantly inhibited cedar pollen-induced conjunctivitis. It has been reported that neither drug showed any inhibition of histamine-induced conjunctivitis.²⁾ However, amlexanox²⁾ and tranilast¹⁴⁾ caused inhibition of egg albumin-induced histamine release in tears. Amlexanox¹⁵⁾ and tranilast¹⁶⁾ have also been reported to inhibit histamine release from rat peritoneal mast cells. Therefore, it is reasonable to presume that inhibition of cedar pollen-induced conjunctivitis by amlexanox and tranilast may be due to inhibition of not only histamine but also the release of other chemical mediators rather than their H₁-antagonistic activity. In conclusion, experimental conjunctivitis in guinea pigs induced by cedar pollen can be useful for both observing allergic response in conjunctival studies and evaluating the effectiveness of antiallergic drugs.

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