Multiple Cedar Pollen Challenge Diminishes Involvement of Histamine in Allergic Conjunctivitis of Guinea Pigs

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It has been reported that antihistamines do not fully modify symptoms of allergic conjunctivitis in clinical settings, suggesting that histamine is not the only contributor to symptom generation in the disease. However, in the majority of experimental allergic conjunctivitis models, antihistamines are very effective in the reduction of symptoms. In the present study, we used our recently developed guinea pig model of allergic conjunctivitis and evaluated whether involvement of histamine in the induction of symptoms of allergic conjunctivitis is altered by multiple antigen challenges. Guinea pigs were sensitized by intraperitoneal injection of Japanese cedar pollen extracts adsorbed on aluminum hydroxide gel, and then challenged by dropping a pollen suspension without the adjuvant on each eye once a week until the 15th challenge. The magnitude of the conjunctivitis intensity score (CIS), itch-associated scratching response and albumin leakage were found to increase with repeated challenges. At the 1st—3rd challenges, histamine H1 receptor antagonist, mepyramine (10 mg/kg, p.o.), strongly reduced all these symptoms. However, symptoms at the 5th—15th challenges were not inhibited by mepyramine. On the other hand, a nitric oxide synthase (NOS) inhibitor, Nω-nitro-L-arginine methyl ester (10 mg/kg, i.v.), potently inhibited the increase of CIS and albumin leakage at the 15th challenge. In conclusion, histamine involvement in the induction of conjunctivitis symptoms in our model was diminished by multiple antigen challenges. The allergic conjunctivitis at the chronic stage is partly mediated by nitric oxide (NO) derived from NOSs that may be activated by mediators other than histamine. The histamine-independent allergic conjunctivitis may be useful for analyzing mechanisms underlying chronic conjunctivitis.

Key words allergic conjunctivitis; histamine; itch; nitric oxide; guinea pig

Allergic conjunctivitis is one of the typical atopic diseases. Recently, a number of patients in Japan have been suffering from Japanese cedar pollinosis with symptoms that include allergic rhinitis and conjunctivitis. The prevalence of allergic conjunctivitis is estimated to occur in approximately 20% of the general Japanese population. The ocular allergy is characterized by severe itching, hyperemia and edema. Medical therapy consists of topical applications of antihistamines with or without vasoconstrictors, mast cell stabilizers, non-steroidal anti-inflammatory drugs and glucocorticoids. Among these treatments, antihistamine is one of the most common medications for allergic conjunctivitis. However, Howarth has suggested that antihistamines do not fully modify the disease since histamine is not the only contributor to symptom generation in allergic conjunctivitis. Despite the existence of a histamine-independent mechanism, in the majority of experimental allergic conjunctivitis models, antihistamines are significantly effective in the reduction of symptoms.

Most experimental ocular allergy animal models have been developed by actively sensitizing guinea pigs with an intraperitoneal injection of a non-airborne antigen such as ovalbumin and dinitrophenylated lysine mixed with an adjuvant or by passively sensitizing animals with antiserum, which is followed by the subsequent single conjunctival challenge with a solution of the specific antigen. These models are considerably different from the clinical situation because the antigen challenges are not performed chronically. To address this situation, we have developed a guinea pig model of allergic conjunctivitis in which sensitized animals are repeatedly challenged by the dropping of a Japanese cedar pollen suspension in the eye. We previously reported that the multiple challenge approach aggravates the disease in proportion to the number of challenges. In the present study, we evaluated whether involvement of histamine in the induction of symptoms of allergic conjunctivitis is altered by the multiple pollen challenges in the model. In addition, in order to know a part of mechanisms underlying the histamine-independent allergic conjunctivitis, we determined whether the symptoms mediated by nitric oxide (NO) synthesis.

MATERIALS AND METHODS

Animals Male, 3-week-old, Hartley guinea pigs weighing 250—300 g were purchased from Japan SLC, Hamamatsu, Japan. The animals were housed in an air-conditioned room at a temperature of 23 ± 1 °C and 60 ± 10% humidity with a controlled 12 h light/dark cycle, and fed a standard laboratory diet with water given ad libitum. The first sensitization was started 1—2 weeks after the purchase. The Experimental Animal Research Committee at Kyoto Pharmaceutical University approved this animal study.

Materials The following reagents were obtained from the indicated commercial sources: mepyramine maleate and L-arginine hydrochloride (Sigma Chem., St. Louis, MO, U.S.A.); Nω-nitro-L-arginine methyl ester (L-NAME) and Nω-nitro-D-arginine methyl ester (D-NNAME) hydrochloride (Wako Pure Chem., Osaka, Japan); guinea pig albumin and anti-guinea pig albumin antibody rabbit serum (Organon Teknika, West Chester, PA, U.S.A.); biotin-N-hydroxysuccinimide and streptavidin-horseradish peroxidase conjugate (Gibco BRL, Gaithersburg, MD, U.S.A.); o-phenylenediamine;
amine (Wako Pure Chem., Osaka, Japan).

In 1998 we personally collected Japanese cedar pollens in the Japanese prefectures of Gifu and Shiga. Al(OH)₃ gels were prepared with 0.5 N NaOH and 0.5 N Al₂(SO₄)₃ as previously described. This method is a modification of the method of Levine and Vaz.

Preparation of Japanese Cedar Pollen Extracts

The cedar pollen extracts used for the sensitization were prepared according to a previously described method. In brief, the pollens were suspended in phosphate-buffered saline at 100 mg/ml and allowed to stand at 4 °C for 18 h under mild stirring. After centrifugation at 1700 × g for 15 min at 4 °C, the resultant supernatant was used as the sensitization antigen.

Sensitization, Challenge, and Treatment with Drugs

As previously described, guinea pigs were sensitized systemically by intraperitoneal injections with the extracts of Al(OH)₃ twice within a week at a dose of 10 μg pollen protein/20 mg Al(OH)₃/ml/animal/time. On the 21st day after the first sensitization, the sensitized animals were challenged by the dropping of a pollen suspension without Al(OH)₃ in each of their eyes at a dose of 2 mg pollens/10 μl/eye. This was followed by repeated challenges with the same dose of antigen once every week until the 15th challenge.

In the first series of experiments a classical histamine H₁ receptor antagonist, mepyramine, was orally administered 1 h before the respective 1st and 15th challenges at 10 mg/kg. In the next experiment, a group of sensitized animals that were different from the first set of experimental animals were orally given mepyramine (10 mg/kg) at the respective 1st—15th challenges. We have previously reported that intraperitoneal or oral administration of 10 mg/kg mepyramine effectively suppressed inductions of early asthmatic response and sneezing in guinea pig models of ovalbumin-induced allergic asthma and cedar pollen-induced allergic rhinitis, respectively.

L-NAME (10 mg/kg, i.v.) and its inactive enantiomer, D-NAME (10 mg/kg, i.v.), were administered 15 min before the 1st or 15th challenge. Furthermore, in order to investigate the reversing effect of L-arginine on L-NAME-induced suppression of allergic responses, L-arginine (600 mg/kg, i.v.) was co-administered with L-NAME at the 15th challenge. In a previous study, we found that intravenous administration of 10 mg/kg L-NAME almost completely suppressed an early phase of nasal blockage, and that the suppression was totally reversed by treatment with L-arginine (600 mg/kg, i.v.) in our Japanese cedar pollen-induced allergic rhinitis model in guinea pigs.

Evaluation of Conjunctivitis

Time course changes of conjunctival edema and redness 0.5—5 h after the 1st, 2nd, 3rd, 5th, 7th, 11th and/or 15th antigen challenges were judged macroscopically. Conjunctivitis intensity score (CIS) was evaluated numerically according to an arbitrary 5-point graded scale from 0 to 4 that increased with severity (0, no symptoms; 1, light; 2, mild; 3, moderate; 4, severe) as per the method of Ballas et al. Additionally, symptoms falling be-

![Fig. 1. Effects of Mepyramine on Increases of Conjunctivitis Intensity Score (CIS, A and D), Amount of Albumin in Ophthalmic Lavage Fluid (B and E) and Scratching Frequency (C and F) at the 1st (A, B and C) and 15th (D, E and F) Challenges in Sensitized Guinea Pigs](image-url)

Mepyramine (10 mg/kg) was given orally 1 h before the respective challenges. ○ and □: Control, ● and ■: Mepyramine. Each point and column represents the mean ± S.E. of 6 or 12 animals. * and **: Statistical significance from the control at p < 0.05 and 0.01, respectively.
between the score 0 and 1 were scored as 0.5.

**Measurement of Scratching Frequency** Scratching frequencies at 0—0.5, 0.5—1 and/or 1—2 h after the respective pollen challenges were counted. The scratch response was defined as an uninterrupted cluster of rapid hind limb movements that were precisely directed to the ocular surface.

**Measurement of Albumin in the Ophthalmic Lavage Fluid** The collection of ophthalmic lavage fluid was carried out as follows: Ten microliters of sterile physiologic saline was applied to the eye using a micropipette (Eppendorf, Hamburg, Germany), without touching the eye. After two or three forced blinks, the lavage fluid was collected. The lavage was repeated 5 times in each eye, and the lavage fluids obtained from both the eyes were combined. The ophthalmic lavage was conducted at 0.5 h after the respective challenges.

Following the centrifugation of the fluid, albumin in the supernatant was measured by an enzyme-linked immunosorbent assay according to the method of Gawin et al.\textsuperscript{18}

**Statistical Analysis** Statistical analyses were performed using the one-way analysis of variance (ANOVA). If a significant difference was detected, the individual group difference was determined by the Bonferroni test for multiple comparisons. A probability value of \( p < 0.05 \) was considered statistically significant.

**RESULTS**

**Effects of Mepyramine on the 1st and 15th Challenge-Induced Conjunctivitis** The CIS that peaked at 0.5 h after challenges, scratching frequency and amount of leaked albumin were found to increase even at the 1st challenge, and the magnitudes of these symptoms intensified up to the 15th challenge (Figs. 1A—F). Mepyramine significantly reduced all these symptoms at the 1st challenge (Figs. 1A—C). However, at the 15th challenge, induced allergic conjunctivitis signs were hardly affected by the histamine H\(_1\) receptor antagonist (Figs. 1D—F).

**Time Course of Changes in Effects of Mepyramine during the Multiple Challenges** In the next series of experiments, we observed time course changes for the effects of mepyramine on the induction of allergic conjunctivitis during the multiple challenges. CIS values at 0.5 h after challenges were increased by the repeated challenges in proportion to the number of challenges during the 1st—5th challenge. Increases of CIS at the 1st—3rd challenges were significantly suppressed by the treatment with mepyramine by over 50%. Mepyramine suppressed the increase of CIS at the 5th challenge, but the degree of inhibition was relatively small. In addition, at the 7th, 11th and 15th challenges, the antihistamine did not reduce the CIS (Fig. 2A).

Albumin leakage at the 1st—3rd challenges was almost completely inhibited by mepyramine, whereas the vascular permeability was not influenced by mepyramine at the 5th—15th challenges (Fig. 2B).

As shown in Fig. 2C, increases in scratching frequency at the 1st—3rd challenges were also significantly reduced by mepyramine by more than 50%. However, the degrees of inhibition by mepyramine on the itch-associated response at the 5th—15th challenges were weaker than those at the 1st—3rd challenges.

**Effects of L-NAME on the Allergic Conjunctivitis**

**Symptoms at the Chronic Stage** In an attempt to discover at least part of the mechanism underlying inductions of allergic conjunctivitis at the chronic stage, we evaluated the effects of a non-selective NOS inhibitor, \( L-\text{NAME} \), on the symptoms induced by the 15th challenge. Our results showed that \( L-\text{NAME} \) significantly inhibited both increases of CIS and albumin amount in the ophthalmic lavage fluid by approximately 60% and 80%, respectively (Figs. 3D, E). However, the increase of scratching frequency was not affected by \( L-\text{NAME} \) (Fig. 3F). The effect of \( D-\text{NAME} \) on these symptoms was negligible (data not shown). The \( L-\text{NAME} \)-induced inhibition of increases of CIS and albumin amount was completely reversed by the co-administration of \( L-\text{NAME} \) with \( L-\text{arginine} \), a substrate of NOS (Figs. 3D, E). Similar to the data for the 15th challenge, there were no significant changes in scratching frequency at the 1st challenge point (Fig. 3C) even though the induced increases of CIS and albumin amount were completely inhibited by \( L-\text{NAME} \) (Figs. 3A, B).

**DISCUSSION**

It has been demonstrated that histamine is one of the
major chemical mediators in allergic conjunctivitis. Indeed, antihistamines effectively suppress all symptoms in patients suffering from allergic conjunctivitis. In addition, in the majority of experimental allergic conjunctivitis models, antihistamines effectively treat the induced symptoms. However, Howarth has reported that antihistamines do not fully modify allergic conjunctivitis in clinical settings, suggesting that mediators other than histamine definitely contribute to the induction. In the present study, we evaluated whether the histamine involvement in the causation of symptoms is altered during multiple pollen challenges in our recently developed guinea pig model for Japanese cedar pollen-induced allergic conjunctivitis.

In most clinical and experimental settings of ocular allergy, pharmacologically active compounds are usually applied as eye drops in order to localize them at a high concentration. However, it is well known that topically applied drugs are rapidly drained into the nasopharyngeal cavity through the nasopharyngeal duct. Indeed, we demonstrated that when Evans blue-Al(OH)₃ was topically applied using eye drops, only 6% of the suspension remained on the conjunctiva at 30 min after the application, indicating that while the concentration of a topically applied drug should be temporarily increased locally at the conjunctiva, most of the drug disappears within 30 min. Thus, in the present pharmacological experiment, we decided to give drugs by systemic administration to maintain a relatively stable concentration in the circulation.

Similar to results of other allergic conjunctivitis models, our first series of experiments in the present study found that the antihistamine mepyramine inhibited the 1st challenge-induced allergic conjunctivitis. Yet, by the 15th challenge, these induced responses were no longer attenuated by the antihistamine. Therefore, we attempted to assess the time course change for the effects of mepyramine during the multiple pollen challenges. As a result, we found a strong reproducible inhibition by mepyramine of the symptoms of allergic conjunctivitis at the 1st—3rd challenges. However at the 5th—15th challenge, the induced conjunctivitis signs were hardly or only slightly affected by the antihistamine. These results strongly suggest that the degree of involvement of histamine in allergic conjunctivitis is altered by the multiple antigen challenge.

All of the conjunctivitis symptoms we observed, i.e., CIS, albumin leakage and scratching behavior, were induced immediately after both the 1st and 15th challenges, suggesting
that mast cell activation largely contributes to the induction of these symptoms at not only the acute but also the chronic stage. However, at present, reasons why the multiple challenges change the involvement of histamine in allergic conjunctivitis are unclear. We can speculate the following reasons may be involved: (1) The releasability of histamine from the mast cells at the conjunctiva may be decreased. (2) The number and/or affinity of histamine H₁ receptors at the local site may be changed.

We have observed a similar phenomenon to the present results in our repeated allergen inhalation challenge-induced asthmatic guinea pig model. Mepyramine was found to significantly inhibit the 2nd challenge-induced early asthmatic response, but this early response induced by the 4th challenge was hardly affected by mepyramine.¹⁴ Different from the present allergic conjunctivitis model, the magnitude of the early asthmatic response was decreased by the repeated inhalation challenge of the antigen.¹⁴ Therefore, in the case of the asthmatic model, it is understandable that the two speculations described above may be related to the diminished involvement of histamine. On the other hand, allergic conjunctivitis symptoms are intensified by the repeated challenge, suggesting that the mechanisms underlying histamine’s diminished involvement in the symptoms are different between the allergic conjunctivitis and asthmatic models. Thus a detailed study of the histamine-independent allergic conjunctivitis at the chronic stage may lead to the finding of new mechanisms underlying chronic allergic conjunctivitis.

We attempted to pharmacologically analyze the mechanisms by using specific or selective antagonists against CysLT₁ receptors, TP receptors, NK₁ and NK₂ receptors, bradykinin B₁ and B₂ receptors, and a cyclooxygenase inhibitor. Unexpectedly, all these compounds failed to suppress the symptoms of allergic conjunctivitis at the chronic stage (Kato et al., unpublished data). Thus, it is unclear which mediators mainly contribute to the induction of the symptoms. However, in the present study, we found that a non-selective NOS inhibitor, L-NAME, inhibited both increases of CIS and albumin at the 15th challenge induced by the 4th challenge was hardly affected by mepyramine. Therefore, in the case of the asthma model, it is understandable that the two speculations described above may be related to the diminished involvement of histamine. On the other hand, allergic conjunctivitis symptoms are intensified by the repeated challenge, suggesting that the mechanisms underlying histamine’s diminished involvement in the symptoms are different between the allergic conjunctivitis and asthmatic models. Thus a detailed study of the histamine-independent allergic conjunctivitis at the chronic stage may lead to the finding of new mechanisms underlying chronic allergic conjunctivitis.

In conclusion, involvement of histamine in inductions of conjunctivitis symptoms in our model is decreased by the multiple antigen challenge. The allergic conjunctivitis at the chronic stage is partly induced by NO derived from NOSs that may be activated by mediators other than histamine. The histamine-independent allergic conjunctivitis may be useful for analyzing mechanisms underlying chronic conjunctivitis.

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