

Inhibitory Effects of Cyclosporine A Eye Drops on Symptoms in Late Phase and Delayed-Type Reactions in Allergic Conjunctivitis Models

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We investigated the efficacy of cyclosporine A (CyA) eye drops on ocular symptoms in late phase and delayed-type reactions in guinea pig allergic conjunctivitis models. An emulsion of ovalbumin (OVA) and Freund's complete adjuvant (FCA) was intraperitoneally injected into guinea pigs, and 15% OVA solution was applied topically to the eyes to elicit late phase reactions. Following the early phase reaction, increased scores for hyperemia, swelling, edema, and discharge were detected 6 h after antigen challenge, and CyA eye drops significantly inhibited the increase in scores for edema and discharge, the increase in the number of infiltrating inflammatory cells, and the percentage of eosinophils among polymorphonuclear leukocytes in conjunctival tissue. To induce delayed-type reactions, guinea pigs were sensitized by injecting FCA into the footpad, followed by injections of purified protein derivative into palpebral conjunctivae 24 d later. Increased scores for hyperemia, swelling, and discharge were detected 6 h after the induction of delayed-type allergy, and CyA eye drops significantly inhibited the increase in scores for hyperemia and swelling. In contrast, betamethasone sodium phosphate eye drops showed a tendency to inhibit the symptoms in both late phase and delayed-type reactions, or inflammatory cell infiltration in the late phase reaction, but the inhibition was not significant. These results suggest that CyA eye drops are useful for suppressing ocular symptoms in both late phase and delayed-type reactions in allergic conjunctivitis models.

Key words cyclosporine A; allergic conjunctivitis; late phase reaction; delayed-type reaction; ocular symptom; inflammatory cell infiltration

Cyclosporine A (CyA) is a cyclic polypeptide calcineurin inhibitor that was first purified in the 1970s by Novartis Pharma (formerly Sandoz Pharma, Switzerland) from cultures of the fungus *Tolypocladium inflatum*. It has long been a trusted agent for suppressing not only graft rejection after transplantation, but also immune reaction disorders often observed in autoimmune diseases. The therapeutic effects of CyA eye drops in the treatment of vernal keratoconjunctivitis (VKC), one of the most severe refractory allergic conjunctival diseases, have been described in several studies.^{1–3)}

VKC involves severe allergic inflammation sufficient to cause the formation of proliferative conjunctival giant papillae, hyperemia, edema, swelling, mucous discharge and corneal damage. It has been reported that the infiltration of inflammatory cells such as mast cells, eosinophils, neutrophils, and T cells were detected in the papillae of VKC patients.^{4–8)} Therefore, in addition to immunoglobulin E (IgE)-mediated allergy induced by mast cells, a late phase reaction mediated by eosinophils and delayed-type allergy induced by T cells are thought to be involved in VKC induction.

Fukushima *et al.* reported the inhibitory effect of CyA eye drops on eosinophil infiltration in a mouse allergic conjunctivitis model.⁹⁾ We have also reported that the instillation of CyA inhibited neutrophil infiltration into the conjunctiva in a delayed-type allergic conjunctivitis model in guinea pigs.¹⁰⁾ However, the efficacy of CyA eye drops in treating the symptoms in these allergic conjunctivitis models has not been previously reported. Therefore, to understand how CyA works in VKC therapy, we investigated the effects of CyA eye drops on ocular symptoms in late phase and delayed-type allergic conjunctivitis models in guinea pigs.

MATERIALS AND METHODS

Reagents CyA was obtained from Novartis Ringaskiddy, Ltd. (Co. Cork, Ireland). Aqueous solution-type CyA eye drops (Papilock Mini[®] ophthalmic solution 0.1%) and the vehicle were prepared at Santen Pharmaceutical Co., Ltd. (Osaka, Japan). Furthermore, 0.01% CyA eye drops were prepared by diluting 0.1% CyA eye drops in the vehicle. Betamethasone sodium phosphate (BP) eye drops (Rinderon[®] solution 0.1%) were purchased from Shionogi Co., Ltd. (Osaka, Japan). Ovalbumin (OVA; Grade V; Sigma, St. Louis, MO, U.S.A.), Freund's complete adjuvant (FCA; Difco, Detroit, MI, U.S.A.), and purified protein derivative (PPD; Nippon-BCG, Tokyo, Japan) were used in the experiments.

Animals Five-week-old male guinea pigs (Hartley, Nippon SLC, Shizuoka, Japan) were housed under a 12-h light–12-h dark cycle (07:00–19:00 h), with room temperature maintained at 23±3 °C and humidity at 50±20%. Food and water were given *ad libitum*. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Santen Institutional Animal Care and Use Committee.

Induction of Late Phase Reaction in the Allergic Conjunctivitis Model Allergic conjunctivitis was induced using the method of Sengoku *et al.*,¹¹⁾ with some modification. OVA solution (40 µg/ml dissolved in saline) was emulsified with an equal volume of FCA. Then, 1 ml of the mixture was intraperitoneally injected into guinea pigs on days 0 and 7. Twenty-four days after the first immunization, 10 µl of 15% OVA solution was applied topically to the right eye to elicit an allergic reaction.

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Induction of the Delayed-Type Allergic Conjunctivitis Model Delayed-type allergic conjunctivitis was induced using the method of Sengoku *et al.*,¹¹⁾ with some modification. Guinea pigs were sensitized by injecting FCA (100 μ l) into the footpad. Fourteen days later, an allergic reaction was elicited by injecting PPD (20 μ g/ml, 30 μ l) into the palpebral conjunctivae of the right eye.

Evaluation of Symptoms To follow the time course of symptoms for each model, extra-ocular symptoms were observed at 0.5, 3, 6, and 24 h after antigen challenge. Four typical symptoms were evaluated: hyperemia in bulbar conjunctiva, swelling in palpebra, edema in bulbar conjunctiva, and discharge. Each symptom was scored as follows: 0, no change; 1, slight change; 2, moderate change; 3, severe change by a well-trained observer to minimize the variation in score.

Drug Instillation and Efficacy Evaluation Ten microliters of drug solution were instilled 3 times into the right eye: 0.5 h before, as well as 2.5 and 5.5 h after antigen challenge. The efficacy of drugs on the symptom scores was evaluated at 6 h. For pathological evaluation of late phase reactions of allergic conjunctivitis, conjunctival tissue was removed 6 h after antigen challenge, fixed with 10% neutralized buffered formalin solution, embedded in paraffin, and cut into 3 μ m-thick vertical sections that included the pupil and optic nerve head. The sections were stained with hematoxylin–eosin and Congo red. Infiltrations of inflammatory cells and eosinophils were evaluated on a graded scale: 0, none or a few inflammatory cells observed in the conjunctiva; 1, some inflammatory cells observed in the conjunctival epithelium and lamina propria; 2, inflammatory cells infiltrating the posterior region along the sclera from the conjunctival region; 3, more inflammatory cells infiltrating than score 2. Eosinophils or polymorphonuclear leukocytes (PMNs) were counted in an area of the lamina propria of the conjunctival sac, including the palpebral and bulbar conjunctiva in each sample.

Statistical Analysis Data from 2 groups were statistically evaluated by the Wilcoxon test or Aspin–Welch's *t*-test. Data from multiple groups were evaluated by Dunnett's multiple comparison test. $p < 0.05$ was considered statistically significant.

RESULTS

Time–Course of Ocular Symptom Scores in the Allergic Conjunctivitis Model Time-dependent changes in ocular symptom scores were evaluated in the allergic conjunctivitis model (Fig. 1). Thirty minutes after antigen challenge, an increase in scores was detected for hyperemia, swelling, edema, and discharge. The scores for edema had at first significantly decreased after 3 h compared to 0.5 h after antigen challenge, but were exacerbated 6 h after the challenge. The score for hyperemia, swelling and discharge did not decrease significantly at 3 h. Thus, in this model, biphasic reactions were detected in the edema score. An increase in all ocular symptom scores continued until 24 h after induction. In all animals, no symptom scores were detected before this experiment.

Time–Course of Ocular Symptom Scores in the Delayed-Type Allergic Conjunctivitis Model Next, time-de-

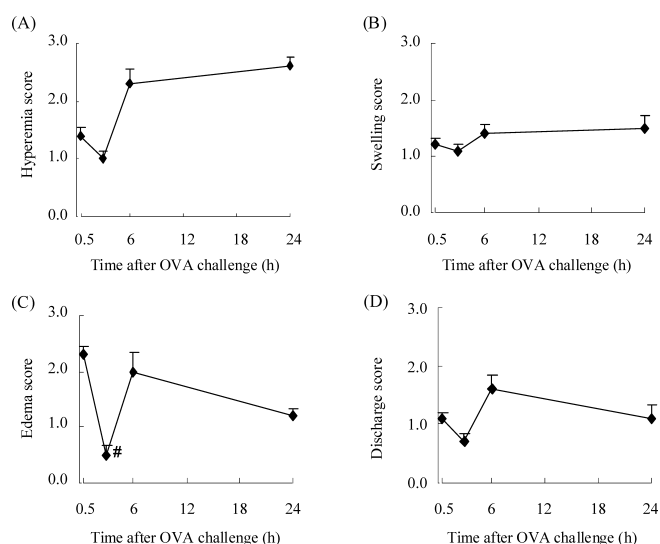


Fig. 1. Time–Course of Ocular Symptom Scores in the Late Phase Reaction of an Allergic Conjunctivitis Model

(A) Hyperemia score, (B) swelling score, (C) edema score, (D) discharge score. Allergic conjunctivitis was induced by OVA challenge in actively sensitized guinea pigs. Ocular symptoms were observed at 0.5, 3, 6, and 24 h after antigen challenge. Data are mean $\pm$ S.E.M. of 10 animals. # $p < 0.05$ compared to 0.5 h (Wilcoxon test).

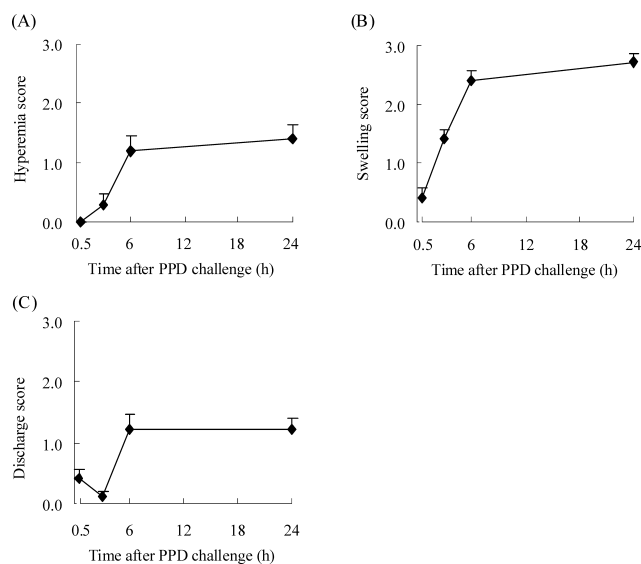


Fig. 2. Time–Course of Ocular Symptom Scores in the Delayed-Type Allergic Conjunctivitis Model

(A) Hyperemia score, (B) swelling score, (C) discharge score. Allergic conjunctivitis was induced by PPD challenge in sensitized guinea pigs. Ocular symptoms were observed at 0.5, 3, 6, and 24 h after antigen challenge. Data are mean $\pm$ S.E.M. of 10 animals.

pendent changes in ocular symptom scores were evaluated in the delayed-type allergic conjunctivitis model (Fig. 2). No increase was detected in the ocular symptom scores within 0.5 h after induction, and none of the scores significantly decreased at 3 h compared with 0.5 h after induction in this model. An increase in scores for hyperemia, swelling, and discharge were detected 6 h after induction, and these scores were maintained until 24 h. No increases in the edema score was detected in this model. In all animals, no symptom scores were detected before this experiment.

Effects of CyA Eye Drops on Ocular Symptom Scores in the Late Phase of the Allergic and Delayed-Type Aller-

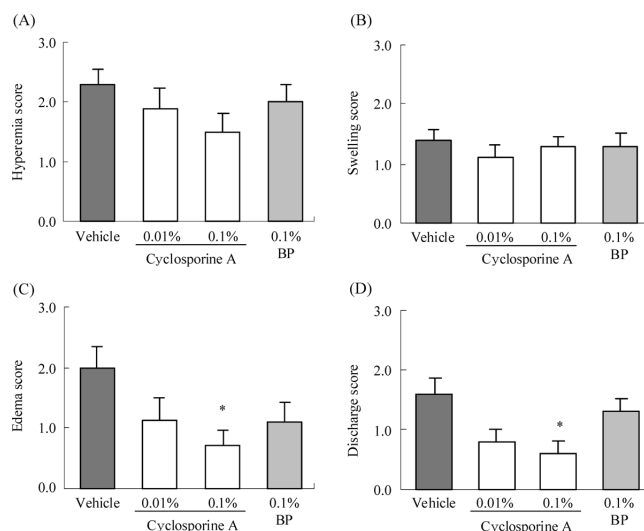


Fig. 3. Effects of CyA and Betamethasone Sodium Phosphate (BP) Eye Drops on Ocular Symptom Scores in the Late Phase Reaction of an Allergic Conjunctivitis Model

(A) Hyperemia score, (B) swelling score, (C) edema score, and (D) discharge score. Allergic conjunctivitis was induced by OVA challenge in actively sensitized guinea pigs. Drug solutions were instilled at 0.5 h before, as well as 2.5 and 5.5 h after, antigen challenge; ocular symptoms were observed at 6 h after antigen challenge. Data are mean $\pm$ S.E.M. of 9 (0.01% CyA) or 10 (others) animals. * p < 0.05 compared to the vehicle group (Dunnett's multiple comparison test).

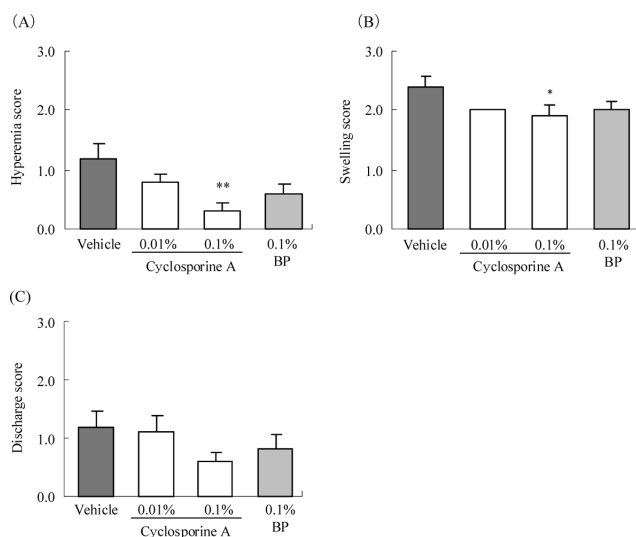


Fig. 4. Effects of CyA and Betamethasone Sodium Phosphate (BP) Eye Drops on Ocular Symptom Scores in a Delayed-Type Allergic Conjunctivitis Model

(A) Hyperemia score, (B) swelling score, and (C) discharge score. Allergic conjunctivitis was induced by PPD challenge in sensitized guinea pigs. Drug solutions were instilled at 0.5 h before, as well as 2.5 and 5.5 h after, antigen challenge; ocular symptoms were observed at 6 h after antigen challenge. Data are mean $\pm$ S.E.M. of 10 animals. * p < 0.05, ** p < 0.01 compared to the vehicle group (Dunnett's multiple comparison test).

gic Conjunctivitis Models The efficacy of CyA eye drops was evaluated for extra-ocular symptom scores at the 6th hour in late phase and delayed-type allergic conjunctivitis models. Instillation of 0.1% CyA significantly inhibited an increase in the scores for edema and discharge compared to those of the vehicle group in the allergic conjunctivitis model (Fig. 3). On the other hand, CyA did not decrease the score for swelling.

In the delayed-type allergic conjunctivitis model, 0.1%

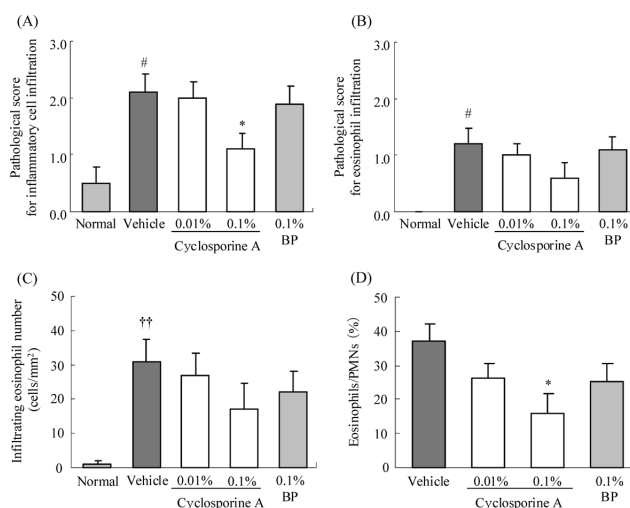


Fig. 5. Effects of CyA and Betamethasone Sodium Phosphate (BP) Eye Drops on Inflammatory Cell Infiltration in the Late Phase Reaction of an Allergic Conjunctivitis Model

(A) Pathological score for inflammatory cell infiltration, (B) pathological score for eosinophil infiltration, (C) infiltrating eosinophil number, and (D) percentage of eosinophils among PMNs. Allergic conjunctivitis was induced by OVA challenge in actively sensitized guinea pigs. Drug solutions were instilled at 0.5 h before, as well as 2.5 and 5.5 h after, antigen challenge; conjunctival tissue was removed at 6 h after antigen challenge for pathological evaluation. Normal group had untreated right eyes. Data are mean $\pm$ S.E.M. of 4 normal or 10 antigen-challenged animals. * p < 0.05 compared to the vehicle group (Dunnett's multiple comparison test); # p < 0.05 compared to the normal group (Wilcoxon test); ** p < 0.01 compared to the normal group (Aspin-Welch t -test).

CyA eye drops significantly decreased the scores for hyperemia and swelling compared to those of the vehicle group, and showed a tendency to decrease the discharge score (Fig. 4). In contrast, although 0.1% BP eye drops showed a tendency to decrease edema, hyperemia, and discharge scores in the late phase reaction allergic conjunctivitis model, and the scores for hyperemia, swelling, and discharge in the delayed-type allergic conjunctivitis model, the inhibitory effects on all symptom scores in both the allergic conjunctivitis models were not significant compared to those of the vehicle group.

Effects of CyA Eye Drops on Inflammatory Cell Infiltration in the Late Phase of the Allergic Conjunctivitis Model The scores for infiltration of inflammatory cells (Fig. 5A) and eosinophils (Fig. 5B) were markedly increased at 6 h after antigen challenge compared to the normal group. Instillation of 0.1% CyA significantly decreased the scores for inflammatory cell infiltration compared to the vehicle group. The number of Congo red-stained eosinophils infiltrating the conjunctiva after antigen challenge was markedly higher than that in the normal group (Fig. 5C). Instillation of 0.1% CyA showed some inhibitory effect on the increase in the number of infiltrating eosinophils compared to that of the vehicle group, although this was not significant.

Next, the percentage of eosinophils among infiltrated PMNs was examined to verify the decrease in eosinophils among PMNs (Fig. 5D). Instillation of 0.1% CyA significantly inhibited the increase in the percentage of eosinophils among PMNs compared to the vehicle group. In contrast, compared to the results of the vehicle group, the inhibitory effects of 0.1% BP eye drops were not significant for the scores for inflammatory cell and eosinophil infiltration, the number of infiltrating eosinophils, or the percentage of eosinophils among PMNs.

DISCUSSION

In IgE-mediated allergy, an early phase reaction is induced by the degranulation of mast cells within a few minutes after antigen exposure. This is followed by a late phase reaction that is characterized by eosinophil infiltration.^{12,13} The early phase reaction initiates allergic conjunctivitis and induces hyperemia, edema, and itching. These symptoms are reproducible in animal models of allergic conjunctivitis, which have been accepted for the evaluation of anti-allergic eye drops.^{14,15} On the other hand, although eosinophil infiltration was observed during the late phase reaction in allergic conjunctivitis models,^{9,16,17} ocular symptoms of the late phase reaction have not been reported.

In this study, symptoms such as hyperemia, swelling, edema, and discharge were induced 30 min after OVA challenge and these are considered the early phase reaction; these symptoms were also elicited at 6 h. In particular, edema scores clearly decreased within 3 h, then increased again at 6 h. Thus, exacerbation of the symptom scores may be considered a late phase reaction of allergy.

Furthermore, 0.1% CyA instillation significantly decreased the scores for edema and discharge at 6 h and showed a tendency to decrease the hyperemia score. However, CyA eye drops did not decrease the score for swelling at 6 h. Once swelling has occurred during the early phase reaction, it may be difficult to show any therapeutic effects of CyA eye drops on swelling in such a short period of time, because swelling might induce tissue damage in the palpebra. In fact, bleeding in palpebra conjunctiva stained with hematoxylin-eosin was observed 6 h after antigen challenge, and CyA eye drops did not show any therapeutic effect on the bleeding (data not shown).

CyA eye drops also decreased eosinophil infiltration and significantly inhibited an increase in the score for inflammatory cell infiltration and the percentage of eosinophils among PMNs at 6 h. Neutrophil infiltration was observed in addition to eosinophil infiltration. In this model, the extent of neutrophil infiltration was almost the same as eosinophil infiltration. However, CyA did not decrease neutrophil infiltration, and in fact the percentage of neutrophils among PMNs was increased after the instillation of CyA eye drops (data not shown). Therefore, instillation of CyA may suppress the symptoms of the late phase reaction, which may be mediated by inhibiting eosinophil infiltration. Those results also suggest that symptoms of the late phase reaction observed in this experiment may be induced by IgE-mediated allergy.

In our preliminary studies with this model, we evaluated the effects of CyA on symptom scores at 30 min after antigen challenge. CyA at 0.1% given 0.5 h before antigen challenge did not show any inhibitory effects on hyperemia, swelling, and discharge (data not shown). This was consistent with our report that showed that the instillation of CyA eye drops 1 h before antigen challenge did not inhibit an early phase reaction in a murine allergic conjunctivitis model.¹⁸ We also reported that instillation of CyA eye drops at 1, 4 and 7 h after antigen-challenge inhibited eosinophil infiltration into conjunctiva in a murine allergic conjunctivitis model.¹⁹ Therefore, it is suggested that the effects of CyA on the late phase reaction are not mediated by suppressing the early phase reaction.

Fukushima *et al.* reported that CyA inhibited eosinophil infiltration, which was induced by an antigen challenge during passive immunization with antigen-primed splenocytes, into the conjunctiva, but failed to inhibit infiltration during passive immunization by IgE transfer in mice.⁹ They concluded that CyA eye drops predominantly inhibited eosinophil infiltration by suppressing T cells in allergic conjunctivitis. We also reported that instillation of CyA after the early phase reaction showed inhibitory effects on the increase in fibrosis scores, collagen content, infiltration by inflammatory cells (CD3⁺ and CD4⁺ T cells, eosinophils), and the expression of interleukin (IL)-4 and IL-5 mRNA in the conjunctiva after 5 d of antigen challenge in a murine allergic conjunctivitis model.¹⁹ These results suggest that the inhibition of T cells may be an important factor in the inhibitory effects of CyA during the late phase reaction in allergic conjunctivitis.

In delayed-type allergy, T cells mediate the activation of inflammatory cells and induce an allergic reaction.^{13,20} In the delayed-type allergic conjunctivitis model of this study, no early phase reaction was detected, as reported in the delayed-type allergic model in rat foot pad,²¹ and an increase in the scores for hyperemia, swelling, and discharge occurred gradually. These increases were observed at 6 h and sustained for up to 24 h after induction. Instillation of CyA significantly decreased the scores for hyperemia and swelling at 6 h in this model. We previously reported that neutrophils were the dominant cell type among the infiltrating inflammatory cells in the delayed-type allergic conjunctivitis model in guinea pigs, and that the instillation of CyA significantly inhibited neutrophil infiltration in this model.¹⁰ Therefore, CyA eye drops may inhibit the symptoms induced by a delayed-type allergy by suppressing neutrophil infiltration mediated by the activation of T cells. It has been reported that CyA eye drops significantly suppressed the scores for objective signs, including swelling, hyperemia, and edema in VKC patients.²² These CyA effects may be mediated by the inhibition of the late phase and delayed-type reactions in clinical situations.

CyA did not decrease neutrophil infiltration in allergic conjunctivitis as described above. Thus, the effects of CyA eye drops on neutrophil infiltration is different between a late phase reaction and a delayed-type reaction. Mast cells have been reported to release IL-8 and interferon (IFN)- γ which are potent chemoattractant factors for neutrophil.^{23,24} Therefore, activation of mast cells in the early phase reaction may be involved in the infiltration of neutrophils into conjunctiva in the allergic conjunctivitis model. CyA was reported to inhibit the release of IFN- γ from both T cells and mast cells at 23 nM and 1 μ M, respectively.^{23–25} Thus, the effects of CyA in mast cells is very weak compared with that in T cells. The different efficacies of CyA between T cells and mast cells may be associated with differences in the inhibitory effects of CyA on neutrophil infiltration between late phase reactions and delayed-type reactions.

In contrast, BP eye drops showed a tendency to inhibit the symptoms in both allergic conjunctivitis models. However, CyA eye drops were more effective than BP eye drops in both these allergic conjunctivitis models. It has been reported that BP inhibited late phase reaction and delayed-type reactions in rat and rabbit, but not in guinea pigs,¹¹ and sensitivity to glucocorticoid was lower in guinea pigs than in other

species.^{26,27)} This low sensitivity to BP in guinea pigs may have been involved in the weak effects of BP eye drops in this study.

We successfully induced ocular symptoms, especially the edema score, in the late phase of an allergic conjunctivitis model. In the delayed-type allergic conjunctivitis model, edema was not detected, although hyperemia and swelling were induced. Thus, edema scores were clearly different between the late phase and delayed-type reactions. Therefore, further study may be needed to understand why only edema formation was different between these two reactions.

In this study, we demonstrated that the instillation of CyA strongly suppressed certain ocular symptoms in late phase and delayed-type reactions in allergic conjunctivitis models. Eosinophil infiltration was detected in the late phase of an allergic conjunctivitis model, and Th2 cytokines such as IL-4 and IL-5 known inducers of eosinophil migration and activation, were present.^{28,29)} Therefore, Th2 cells may be involved in the induction of the late phase reaction. In contrast, neutrophils were the dominant cell type among infiltrating inflammatory cells in the delayed-type allergic conjunctivitis model in our previous experiments.¹⁰⁾ IFN- γ , a typical Th 1 cytokine, has been reported to induce neutrophil migration,³⁰⁾ which suggests that Th1 cells may mediate symptom induction in the delayed-type reaction. It had been reported that CyA inhibited Th1 and Th2 cytokine production in *in vitro* studies, and the concentration of CyA after instillation of 0.05% CyA eye drops was sufficient to show an inhibitory effect on T cell mediated cytokine production in conjunctiva.²⁵⁾ Therefore, CyA eye drops may suppress ocular symptoms that are mediated by both Th1 and Th2 cells in conjunctivitis.

Acknowledgments We thank Satsuki Yamaguchi for technical assistance.

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