

Effect of FK506 eye drops on late and delayed-type responses in ocular allergy models

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Summary

Background It is well-known that FK506 strongly inhibits cytokine production by T cells *in vitro*. However, less evidence is available from *in vivo* studies of ocular allergy.

Objective To study the anti-inflammatory effect of FK506 eye drops on late and delayed-type responses in several animal models of ocular allergy.

Methods Rats and guinea-pigs were sensitized with egg albumin (EA) in adjuvant and later challenged by topical EA application to their eyes to examine the late response. Biopsy specimens of conjunctiva were stained with haematoxylin–eosin or stained for T cells and eosinophils. In addition, rats, rabbits and guinea-pigs were sensitized with complete Freund's adjuvant and later challenged by injecting purified protein derivatives for the delayed-type response. Bulbar conjunctival oedema and hyperaemia were graded by score in rabbits, and Evans blue (EB) extravasation was measured in rats and guinea-pigs. FK506 (0.01–1%) and steroid (0.1%) eye drops were instilled in the eyes of animals several times, before and after challenge.

Results FK506 eye drops inhibited T cell and eosinophil infiltration in the late response and EB extravasation in the delayed-type response in rats. Also, they inhibited conjunctival oedema, hyperaemia and ocular mucus in the delayed-type response in rabbits. These effects were similar to those of steroid eye drops (betamethasone sodium phosphate, fluorometholone). FK506 eye drops also inhibited inflammatory cell infiltration, the loss of conjunctival epithelium and decrease of goblet cells in the late response as well as EB extravasation in the delayed-type response in guinea-pigs, a steroid-resistant species.

Conclusion FK506 eye drops inhibit late and delayed-type responses in animal models of ocular allergy.

Keywords allergic conjunctivitis, corticosteroids, delayed-type hypersensitivity, FK506 eye drops, guinea-pigs, late response, ocular allergy models, rabbits, rats

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Introduction

The most common ocular allergy is allergic conjunctivitis. It is classified on the basis of clinical criteria into seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) [1]. Until recently, it was thought that allergic conjunctivitis was an acute conjunctival inflammation induced through immediate-type responses triggered by mast cells sensitized with IgE. However, increasing attention has been devoted to the fact that clinical signs of allergic conjunctivitis are not transient and last for a period from several hours to several days [2]. This prolonged conjunctival inflammation occurs via a late response, as evident from the infiltration of inflammatory cells, such as

eosinophils, neutrophils and Th2 cells, into the conjunctiva following the immediate-type response, and is maintained by networks of several cytokines. In contrast, in chronic VKC and AKC, the delayed-type response in addition to immediate-type and late responses, can lead to more serious damage, such as vision-threatening conditions due to corneal damage [3]. This is due to complicated interactions of various types of inflammatory cells, such as mast cells, eosinophils, neutrophils, Th2 or Th1 cells and Langerhans cells, which increase in number in the conjunctiva, and to cytokines liberated from these cells [4, 5]. Therefore, some types of allergic conjunctivitis have been proposed to be induced by the late and delayed-type responses in which Th2 and Th1 immune responses, respectively, are important [6].

In the light of the therapy, weak or mild cases of SAC and PAC are usually treated with an anti-allergic eye drop that inhibits histamine release by mast cells. However, a steroid eye drop is the only therapy available for treatment of severe cases of SAC and PAC, as well as for the treatment of incurable and debilitating disorders such as chronic VKC

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and AKC [7]. However, because of the severe side-effects of steroids [8], the development of a new drug that possesses high efficacy/low side-effect characteristics is required.

FK506 (tacrolimus hydrate) [9] is a novel macrolide immunosuppressant discovered in 1984 by the Exploratory Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan) and is now used for transplantation worldwide. Outside of the transplantation field, FK506 ointment [10–12] is currently available for treatment of atopic dermatitis in some countries, including the USA, Canada and Japan. FK506 ointment has a high efficacy and fewer side-effects compared to steroid ointments. FK506 strongly inhibits cytokine production by T cells *in vitro* [13–16] and FK506 ointment suppresses delayed-type cutaneous responses in mice, by inhibiting the activation of sensitized Th1 cells already accumulated in the dermis [17]. Although FK506 inhibits histamine release and cytokine production by mast cells and basophils *in vitro* [18–20], FK506 ointment does not suppress the immediate-type response in rats [17].

In this paper, we focused for the first time on the effects of FK506 eye drops (0.01–1%) on the late and delayed-type responses in several animal models of ocular allergy, because both T cell-mediated responses are most important in severe and chronic cases of allergic conjunctivitis [4, 5, 21]. Here, we compared the effects of FK506 eye drops with those of 0.1% steroid eye drops (betamethasone sodium phosphate, fluorometholone), which have been used in severe and chronic cases of allergic conjunctivitis.

Materials and methods

Animals

Male Brown Norway rats (8 weeks old; Nippon Charles River, Hino, Japan), male Hartley guinea-pigs (3 weeks old; Nippon SLC, Hamamatsu, Japan), male Lewis rats (7 weeks old; Nippon Charles River) and male New Zealand White rabbits (8 weeks old; Kitayama Rabesu, Minowa, Japan) were used. They were allowed to adapt to the environment and maintained on a standard diet and water *ad libitum*. The animals were handled in adherence to Fujisawa Pharmaceutical Animal Experiment Committee guidelines.

Eye drops

FK506 eye drops (ophthalmic suspension; 0.01–1%) and 0% FK506 eye drops were prepared at Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). Betamethasone sodium phosphate eye drops (0.1%, Rinderon[®]; Shionogi, Osaka, Japan) and fluorometholone eye drops (0.1%, Flumetholon[®]; Santen, Osaka, Japan) were purchased.

Late response in rats

Egg albumin solution (EA; Seikagaku Kogyo, Tokyo, Japan) (2 mg/mL) was mixed with an equal volume of 10 mg/mL of aluminium hydroxide hydrate gel suspension (ALUM; LSL, Tokyo, Japan), then 1 mL of the mixture and 1 mL of the adjuvant *Bordetella Pertussis* (6×10^9 cells/mL; Kaken Seiyaku, Tokyo, Japan) were injected subcutaneously and intraperitoneally, respectively, into Brown Norway rats for sensitization. Two weeks later, 100 mg/mL of EA (10 μ L

each) was topically applied to both eyes to elicit the late response in the conjunctiva. Rats were killed by ether anaesthesia and exsanguination 24 h after challenge and then histological analysis was performed. The right conjunctival tissues containing the eyeball were removed and fixed in 10% buffered formalin solution, embedded in paraffin by conventional methods, cut in 3 μ m sections and stained with haematoxylin–eosin (HE). The conjunctival inflammation score characterized by infiltration of inflammatory cells was graded with a microscope according to the following criteria: no change, 0; slight change, 1; moderate change, 2; marked change, 3; and very strong change, 4. Scoring was performed by two trained observers. The left conjunctival tissues containing the eyeball were removed and embedded in OCT compound (Miles, Inc., Elkhart, IN, USA), snap frozen in dry ice–acetone, and stored at -80°C until use. Frozen sections, cut in 5 μ m slices, were fixed in acetone for 10 min. After pre-treatment with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase, the preparations were washed with phosphate-buffered saline (PBS) twice and treated with blocking medium (10% normal goat serum in PBS). Mouse monoclonal antibody against rat $\alpha\beta$ -T cell receptor (TCR) (Pharmingen, San Diego, CA, USA) was then applied for 1 h in PBS with 1% bovine serum albumin, and after washing, goat anti-mouse IgG conjugated with peroxidase (Jackson, West Grove, PA, USA) was overlaid for 30 min. Visualization for the reaction products was performed with 3-amino-9-ethyl-carbazole. For staining of eosinophils, the frozen sections were treated with *o*-phenylene diamine after fixation using 10%-buffered formalin. The number of T cells and eosinophils infiltrating the conjunctiva in each section was counted with a microscope. Each drug (20 μ L divided into four parts) was instilled in both eyes five times, 3 and 1 h before, and 2, 4 and 21 h after challenge.

Delayed-type response in rats

Lewis rats were sensitized by subcutaneous injections of complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA) (0.6 mL/head). Two weeks later, delayed-type response was elicited by injection of purified protein derivative (PPD; Nippon-BCG, Tokyo, Japan) (50 μ g/mL, 10 μ L each) into the upper and lower palpebral conjunctivae of both eyes. Evans blue (EB; Seikagaku Kogyo) solution (0.5%, 1 mL) was injected intravenously 20 h after challenge and the amount of dye leaking into the palpebral conjunctivae of both eyes, over 4 h, was determined using a spectrophotometer. Each drug (20 μ L divided into two parts) was instilled in both eyes four times, 2 h and just before, and 2 and 4 h after challenge.

Late response in guinea-pigs

EA (20 mg/mL) was mixed with an equal volume of CFA, and 1 mL of the mixture was injected intraperitoneally into guinea-pigs for sensitization. Twenty-three days later, 100 mg/mL of EA (20 μ L each) was topically applied to both eyes to elicit the late response in the conjunctiva. Guinea-pigs were killed by ether anaesthesia and exsanguination 6 h after challenge. The conjunctival tissues containing the eyeball of both eyes were stained with HE. The conjunctival inflammation score, mainly characterized by infiltration of

inflammatory cells, was graded with a microscope according to the following criteria: no change, 0; slight change, 1; moderate change, 2; marked change, 3; and very strong change, 4. Scoring was performed by two trained observers. Each drug (20 μ L) was instilled in both eyes three times, 4 and 2 h before, and 2 h after challenge.

Delayed-type response in guinea-pigs

Guinea-pigs were sensitized by subcutaneous injections of CFA (0.6 mL/head). Two weeks later, delayed-type response was elicited by injection of PPD (200 μ g/mL, 10 μ L each) into the upper and lower palpebral conjunctivae of both eyes. EB solution (1%, 1 mL) was injected intravenously 20 h after challenge and the amount of dye leaking into the palpebra of both eyes, over 4 h, was determined using a spectrophotometer. Each drug (20 μ L) was instilled in both eyes three times, 4 and 2 h before, and 3 h after challenge.

Delayed-type response in rabbits

Rabbits were sensitized by subcutaneous injections of CFA (6 mL/head). Two weeks later, delayed-type response was elicited by injection of PPD (10 μ g/mL, 30 μ L each) into the upper bulbar conjunctiva of both eyes. At 24 h after challenge, the bulbar conjunctival oedema and hyperaemia in both eyes, respectively, were scored on a scale of 0–3, according to the following criteria: no change, 0; slight change, 1; moderate change, 2; and marked change, 3. Scoring was performed by two trained observers. The sum of conjunctival oedema score plus hyperaemia score gave the conjunctival inflammation score. Immediately after scoring, the physical appearance of the eye, including the periocular region, in a representative rabbit from each group was photographed. Each drug (30 μ L) was instilled in both eyes four times a day (two times on the final day), starting at 3 days before challenge (total 18 times).

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was carried out using Tukey–Kramer's or Dunnett's multiple comparison test, following Kruskal–Wallis test (in the case of scores) or one-way ANOVA. *P*-values less than 0.05 were considered statistically significant.

Results

Effects on late response in rats

We first compared the effects of FK506 eye drops with those of steroid eye drops on the late response in rats. In this model, at 24 h after challenge with EA, the conjunctival inflammation score was significantly increased in the sensitized and challenged mice (control mice), compared with normal mice (Fig. 1a). Furthermore, T cell and eosinophil infiltrations in the conjunctiva were significantly increased in control mice, compared with normal mice (Figs. 1b and c). These T cell and eosinophil infiltrations were consistent with the conjunctival inflammation score. FK506 eye drops inhibited not only conjunctival inflammation score (1%, $P < 0.01$) (Fig. 1a) but also the increase of inflammatory cells such as T cells (0.1–1%, $P < 0.01$) (Fig. 1b) and eosinophils (0.3%, $P < 0.05$; 1%, $P < 0.01$) (Fig. 1c) in a dose-dependent manner. Although two kinds of steroid eye drops (betamethasone sodium phosphate eye drops (0.1%) and fluorometholone eye drops (0.1%)) showed significant suppressive effects on T cell and eosinophil infiltrations to the same degree as FK506 eye drops (Figs. 1b and c), they did not significantly inhibit the conjunctival inflammation score (Fig. 1a).

Effects on delayed-type response in rats

An apparent increase in the EB dye leakage based on delayed-type response was found in sensitized rat conjunctiva challenged with PPD. FK506 eye drops showed a dose-dependent significant suppressive effect on EB extravasation (0.03–0.3%, $P < 0.01$) (Fig. 2). Two kinds of steroid eye drops (betamethasone sodium phosphate eye drops (0.1%) and fluorometholone eye drops (0.1%)) showed suppressive effects to the same degree as FK506 eye drops (Fig. 2).

Effects on late response in guinea-pigs

Guinea-pigs in the control group exhibited dramatic changes in histology at 6 h after challenge. One of the most striking features was the inflammatory cell infiltrate, but in addition, the loss of conjunctival epithelium and decrease in the number of goblet cells were also evident.

The conjunctival inflammation score, mainly graded by the degree of inflammatory cell infiltrate, was significantly increased in the control group compared to either the normal,

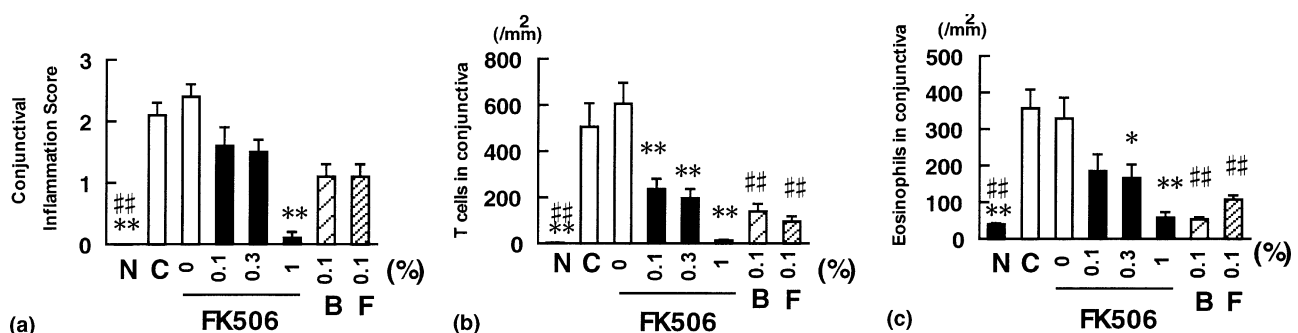


Fig. 1. Effects of FK506 eye drops and steroid eye drops on the conjunctival inflammation score (a), T cell infiltrate (b) and eosinophil infiltrate (c) in the conjunctiva in the late response in rats. N, normal group; C, sensitized and challenged control group; B, betamethasone sodium phosphate eye drops; F, fluorometholone eye drops. $n = 10$ per group, * $P < 0.05$, ** $P < 0.01$, compared with FK506 (0%), ## $P < 0.01$, compared with C, by Tukey–Kramer's test.

unchallenged or unsensitized group (Fig. 3). FK506 eye drops showed potent suppressive effects on conjunctival inflammation score (0.03%, 0.1% and 1%, $P < 0.05$; 0.3%, $P < 0.01$) (Fig. 3). In addition, conjunctival epithelium and goblet cells in guinea-pigs treated with FK506 eye drops were almost normal in appearance, that is, FK506 eye drops inhibited the

loss of conjunctival epithelium and decrease in the number of goblet cells (data not shown).

Effects on delayed-type response in guinea-pigs

An apparent increase in the EB dye leakage was found in sensitized guinea-pig palpebra challenged with PPD. FK506 eye drops showed a dose-dependent significant inhibitory effect on EB extravasation (0.1–1%, $P < 0.01$) (Fig. 4a). In contrast, betamethasone sodium phosphate eye drops (0.1%) did not significantly inhibit EB extravasation in this model (Fig. 4b).

Effects on delayed-type response in rabbits

Sensitized and challenged control rabbits showed clinical features characterized by bulbar conjunctival oedema and hyperaemia at 20 h after challenge. Conjunctival inflammation score, defined as the sum of conjunctival oedema score and hyperaemia score, was apparently increased in control rabbits (score in normal rabbits = 0) (Fig. 5). FK506 eye drops showed a significant suppressive effect on conjunctival inflammation score (0.01%, $P < 0.05$; 0.03% and 0.1%, $P < 0.01$) (Fig. 5). Steroid eye drops (betamethasone sodium phosphate eye drops (0.1%) and fluorometholone eye drops (0.1%)) showed suppressive effects to the same degree as FK506 eye drops (Fig. 5). Photographs of the eye, including the periocular region, in a representative rabbit from each group showed that FK506 eye drops and steroid eye drops inhibited not only conjunctival oedema and hyperaemia but also ocular mucus (Fig. 6).

Discussion

In this paper, we studied for the first time the effects of FK506 eye drops (0.01–1%) on the late and delayed-type responses in several animal models of ocular allergy, compared with those of steroid eye drops.

In rats, FK506 eye drops inhibited the conjunctival late and delayed-type responses to the same degree as steroid eye drops. Also, in the delayed-type response of rabbits, FK506 inhibition of the conjunctival oedema, hyperaemia and ocular mucus was clearly visible to the naked eye to the same degree as steroid eye drops. These data suggest that FK506 eye drops

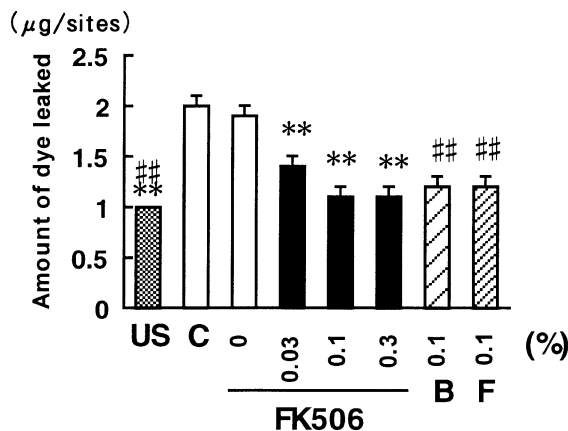


Fig. 2. Effects of FK506 eye drops and steroid eye drops on EB extravasation in the delayed-type response in rats. US, unsensitized group; other abbreviations are defined in Fig. 1. $n = 5$ per group, ** $P < 0.01$, compared with FK506 (0%), ## $P < 0.01$, compared with C, by Tukey–Kramer's test.

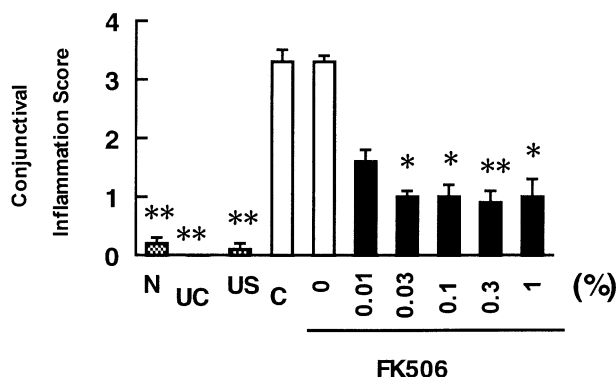


Fig. 3. Effects of FK506 eye drops on the conjunctival inflammation score in the late response in guinea-pigs. UC, unchallenged group; other abbreviations are defined in Figs. 1 and 2. $n = 10$ per group, * $P < 0.05$, ** $P < 0.01$, compared with FK506 (0%), by Dunnett's test.

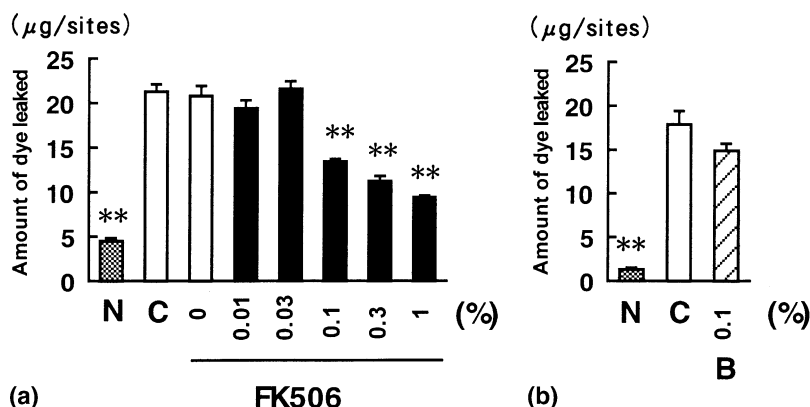


Fig. 4. Effects of FK506 eye drops (a) and steroid eye drops (b) on EB extravasation in the delayed-type response in guinea-pigs. The abbreviations are defined in Fig. 1. (a) $n = 7$ per group, ** $P < 0.01$, compared with FK506 (0%), by Dunnett's test. (b) $n = 8$ per group, ** $P < 0.01$, compared with C, by Dunnett's test.

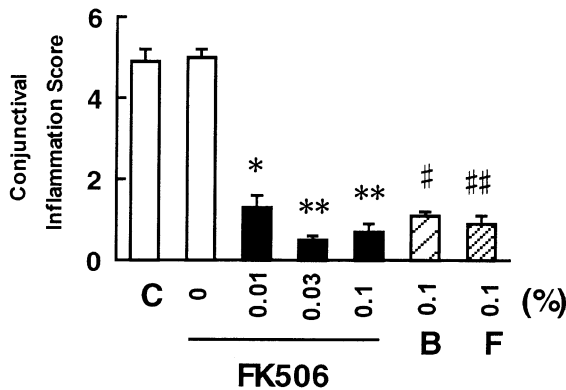


Fig. 5. Effects of FK506 eye drops and steroid eye drops on the conjunctival inflammation score in the delayed-type response in rabbits. The abbreviations are defined in Fig. 1. $n = 11$ – 12 per group, * $P < 0.05$, ** $P < 0.01$, compared with FK506 (0%), # $P < 0.05$, ## $P < 0.01$, compared with C, by Tukey–Kramer's test.

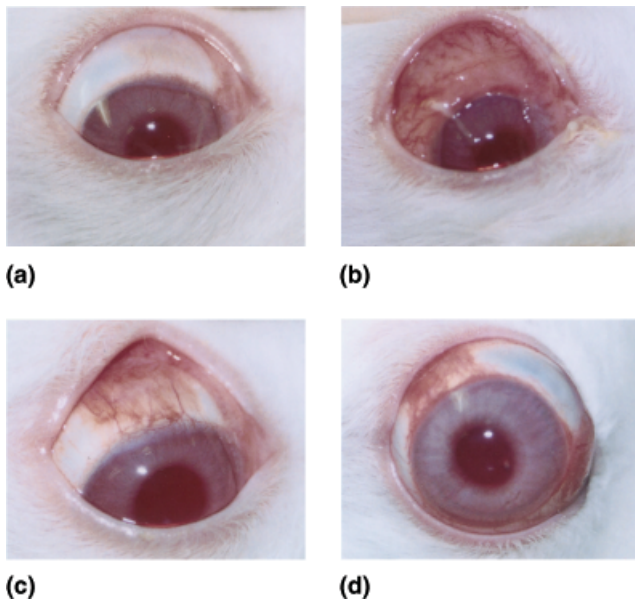


Fig. 6. Eye of rabbit (a) in normal condition, (b) sensitized and challenged control condition treated with FK506 (0%) eye drops, (c) treated with FK506 (0.1%) eye drops and (d) treated with betamethasone sodium phosphate (0.1%) eye drops. FK506 eye drops and steroid eye drops inhibited conjunctival oedema, hyperaemia and ocular mucus (compare (c) and (d) with (b)).

might have potential for a clinically relevant effect in human ocular allergy, as the suppressive effects of FK506 eye drops on the conjunctival late and delayed-type responses were equivalent to those of steroid eye drops.

Prolonged topical use of steroids may lead to steroid-specific optic adverse reactions such as secondary infection, glaucoma or cataracts in humans [3]. Therefore, it is recommended to avoid using steroid eye drops for a prolonged period. However, in pre-clinical animal studies, FK506 eye drops showed no severe optic side-effects (data not shown). Thus, FK506 eye drops may have potential as a novel drug that possesses strong efficacy similar to steroid eye drops and minimizes optic side-effects.

However, there are some steroid-resistant patients with ocular allergy [22]. Therefore, we next studied the effects of

FK506 eye drops on the conjunctival late and delayed-type responses in guinea-pigs, to determine the effect of FK506 in a steroid-resistant species. A recent report [23] shows that guinea-pigs exhibit resistance to steroids because the guinea-pig steroid receptor (GR) has a lower affinity for cortisol than the human GR. Although cellular mechanisms contributing to inadequate response to steroid therapy are not clear in ocular allergy, they have been recently clarified in human steroid-resistant asthma. In steroid-resistant asthma, resistance appears to be related to reduced GR-binding affinity in T cells (like the resistance mechanism in guinea-pigs) as well as reduction in GR number [24, 25]. It has also been reported that T cells from steroid-resistant asthma patients are significantly less inhibited by steroids compared with T cells from steroid-sensitive asthma patients. In contrast, FK506 inhibited T cells both from steroid-sensitive and -resistant patients with equivalent potency [26]. In this paper, FK506 eye drops, but not betamethasone sodium phosphate eye drops, inhibited the delayed-type response in guinea-pigs. This result suggests that FK506 inhibits ocular allergy in guinea-pigs, a steroid-resistant species, through mechanisms independent of GR. Although data were not shown, when we studied the effects of some anti-allergic eye drops (2% sodium cromoglycate, 0.5% tranilast, 0.25% amlexanox and 0.1% pemirolast potassium) that are known to be unsatisfactory for treatment of chronic ocular allergies, such as VKC and AKC, they did not inhibit the delayed-type response in guinea-pigs, consistent with clinical results. In addition, cyclosporin A in olive oil, which has sometimes been used for steroid-resistant chronic ocular allergy as a substitute for steroids [27], showed only a weak inhibition of the delayed-type response in guinea-pigs at a concentration of 3% (data not shown). Thus, FK506 eye drops might have potential as the only therapy available for the treatment of steroid-resistant patients with chronic ocular allergy, although clinical trials of FK506 eye drops are needed.

Furthermore, FK506 eye drops inhibited the late-phase response in guinea-pigs. An interesting result obtained in this experiment is that FK506 eye drops inhibited inflammatory cell infiltration and also inhibited the loss of conjunctival epithelium and decrease in the number of goblet cells. The loss of conjunctival epithelium and decrease in the number of goblet cells, which play an important role in mucus secretion, lead to the destruction of barrier function in the conjunctiva. For the protection of barrier function in the eyes, FK506 might lead to normalization of the allergen-exclusion system.

In conclusion, our study showed that FK506 eye drops inhibited late and delayed-type responses in animal models of ocular allergy. As the FK506 action mechanism is thought to be mainly based on inhibition of cytokine production by T cells [13–16], this suggests that FK506 eye drops inhibit the late and delayed-type responses in conjunctiva by suppressing Th2 cytokine and Th1 cytokine production by T cells, respectively.

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