
LABORATORY INVESTIGATION

Comparison of Topical Dexamethasone and Topical FK506 Treatment for the Experimental Allergic Conjunctivitis Model in Balb/c Mice

Jun Shoji¹, Tohru Sakimoto¹, Keiko Muromoto¹, Noriko Inada¹, Mitsuru Sawa¹, and Chisei Ra²

¹Department of Ophthalmology, Nihon University School of Medicine, Tokyo, Japan;

²Division of Molecular Cell Immunology and Allergology, Nihon University Graduate School of Medical Sciences, Tokyo, Japan

Abstract

Purpose: To evaluate the efficacy of topical dexamethasone and topical FK506 treatment on allergic inflammation in conjunctiva, we performed a comparative study using a mouse experimental allergic conjunctivitis model.

Methods: The experimental allergic conjunctivitis model was developed by ovalbumin (OVA) instillation after OVA sensitization by intraperitoneal application of a mixed solution of OVA and aluminum hydroxide. The Balb/c mice of the experimental allergic conjunctivitis model were divided into three groups, depending on whether or not they received topical treatment and which type of treatment they received. The allergy group comprised six mice without topical treatment; the dexamethasone (Dx) group comprised six mice receiving topical 0.1% dexamethasone ointment treatment; and the FK506 (FK) group comprised six mice receiving topical 0.1% FK506 ointment treatment. The negative control group comprised six mice with neither sensitization nor topical treatment. For the histological examination, frozen sections of the eyelids, eyeballs, and lacrimal glands were stained with acid Giemsa stain or, in the immunohistochemical method, with alkaline phosphatase–antialkaline phosphatase (APAAP), for CD4. Densities of eosinophils and CD4-positive lymphocytes in the conjunctival tissue were counted. Productive *C ε* gene expression in the conjunctival tissue, including the lacrimal gland, was also evaluated by reverse transcriptase polymerase chain reaction (RT-PCR).

Results: In the histological study, the cell density of eosinophils infiltrating into subconjunctival tissues was 66.3 ± 34.7 cells/mm² (mean $\pm$ SD) for the allergy group, 2.5 ± 4.2 cells/mm² for the Dx group, 4.2 ± 8.6 cells/mm² for the FK group, and 3.7 ± 7.6 cells/mm² for the negative control group. The cell density of CD4-positive cells infiltrating into subconjunctival tissues was 145.9 ± 66.4 cells/mm² for the allergy group, 10.0 ± 12.9 cells/mm² for the Dx group, 10.1 ± 15.5 cells/mm² for the FK group, and 21.7 ± 17.8 cells/mm² for the negative control group. The allergy group showed significant differences in the density of eosinophils and CD4-positive cells compared with those of the Dx ($P < 0.0001$) and FK ($P < 0.0001$) groups. In the RT-PCR study, the expression of productive *C ε* gene was positive in the Dx group, but negative in the allergy group, the FK group, and the negative control group.

Conclusions: Topical Dx and FK506 treatments were both efficacious in suppressing eosinophil and lymphocyte infiltration into subconjunctival tissue in the mouse experimental allergic conjunctivitis model. However, the influence of their IgE productive mechanisms seemed to differ, causing different efficacy of productive *C ε* gene expression in the conjunctival tissue. **Jpn J Ophthalmol** 2005;49:205–210
© Japanese Ophthalmological Society 2005

Key Words: allergic conjunctivitis, Balb/c mice, *C ε* gene, dexamethasone, eosinophil, FK506

Received: May 24, 2004 / Accepted: October 21, 2004

Correspondence and reprint requests to: Jun Shoji, Department of Ophthalmology, Nihon University School of Medicine, 30-1 Oyaguchi-kamimachi, Itabashi-ku, Tokyo 173-8610, Japan
e-mail: shoji@med.nihon-u.ac.jp

Introduction

In allergic conjunctival disease (ACD), allergic inflammation characterized by infiltration of eosinophils and lym-

phocytes mainly into the conjunctiva and elevation of antigen-specific IgE levels in serum and tears are very important factors to consider when developing new treatments. Allergic inflammation is considered to contribute to exacerbation of the pathophysiology of severe ACD, for example, vernal keratoconjunctivitis (VKC).¹ Instillation, subconjunctival injection, or systemic administration of corticosteroids (steroids) have been employed to treat the allergic inflammation of ACD. However, steroid treatment may have potent adverse events such as steroid-induced glaucoma and corneal infections. Topical administration of immunosuppressive drugs such as cyclosporine A and FK506 was introduced to provide a drug with a potency equal to steroids with less adverse effect. Instillation of cyclosporine A for VKC has been reported to be effective for alleviating clinical symptoms.^{1–4} FK506 is a new macrolide immunosuppressive agent extracted from the soil bacterium *Streptomyces tsukubaensis*. Regarding its efficacy against allergic reaction, it is known to inhibit production of cytokines in T cells^{5,6} and histamine release from mast cells.⁷ FK506 (Protopic) has been reported to be effective on a rat experimental allergic blepharoconjunctivitis model⁸ and in patients with atopic blepharoconjunctivitis.⁹ However, the therapeutic mechanism of these drugs for alleviating the clinical manifestation of ACD still remains unclear.

Using a mouse experimental allergic conjunctivitis model, we performed a comparative study between topical dexamethasone and FK506 treatment with respect to the infiltration of eosinophils and lymphocytes and the expression of the productive *C ε* gene associated with IgE production in the conjunctiva.

Materials and Methods

Mouse Model for Experimental Allergic Conjunctivitis and the Drug Instillation Method

Balb/c mice (24 males), aged 8 to 12 weeks, were obtained from Oriental Yeast (Tokyo, Japan). All the animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) resolution regarding animals used in research. The mice were divided into the following four groups: the allergy group (positive control), dexamethasone group (Dx group), FK506 group (FK group), and the negative control group. Each group comprised six mice.

Experimental allergic conjunctivitis in the mice was developed by the following method (Fig. 1). On the first day, a 0.2-ml mixed solution, adjusted to a concentration to 5 µg/ml of ovalbumin (OVA) and 15 mg/ml of aluminum hydroxide, was administered intraperitoneally. Additionally, on day 8, the same intraperitoneal treatment was done for sensitization. On day 15, an OVA solution (5 mg/ml) was instilled into both eyes. On day 18, an OVA solution was again instilled into both eyes to induce allergic conjunctivitis. From day 15 to day 18, the mice in the Dx and FK groups were treated with topical dexamethasone (Dx) or FK506.

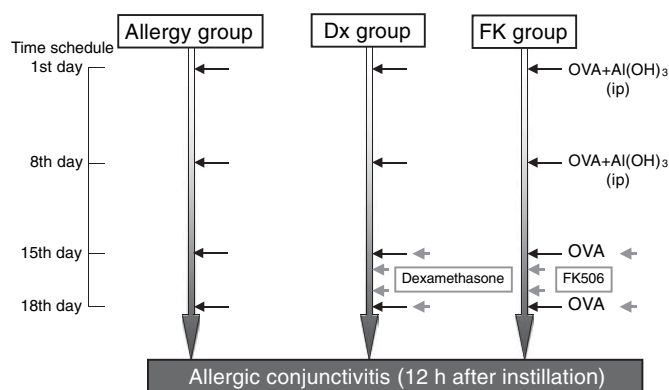


Figure 1. The protocol for the experimental allergic conjunctivitis and medical therapy. Dx group, dexamethasone group; FK group, FK506 group; OVA, ovalbumin; Al (OH)₃, aluminium hydroxide; ip, intraperitoneal administration.

The drugs used were 0.1% dexamethasone eye ointment (Santezone; Santen, Osaka, Japan) in the Dx group and 0.1% FK506 ointment (Protopic; Fujisawa Pharmaceutical, Osaka, Japan) in the FK group. Either dexamethasone or FK506 was instilled once a day for 4 days. The negative control group comprised mice without sensitization or topical treatment. Twelve hours after the final instillation of OVA solution, eyeballs, eyelids, and lacrimal glands were removed from the mice of all groups, after euthanasia by pentobarbital (Nembutal; Dainippon Pharmaceutical, Osaka, Japan). Histological examination was performed using one eye from each animal, and the molecular biological examination was done using the fellow eye.

Histological Study

The removed eyeballs and eyelids were fixed in a periodate-lysine-paraformaldehyde solution for 2 h, embedded in OCT compound (Tissue-Tek, Sakura Seiki, Tokyo, Japan), and rapidly frozen in dry ice-isopentane. The frozen sample was cut into approximately 7-µm-thick frozen sections with a cryostat (HM505E, Microm International GmbH, Walldorf, Germany). The sections were stained with acid Giemsa stain (DiffQuick staining kit, Kokusai Shiyaku, Kobe, Japan) or by the alkaline phosphatase–antialkaline phosphatase (APAAP) method for immunohistochemical analysis. The first antibody used in the APAAP method was anti-mouse CD4 rat monoclonal antibody (Clone; CT-CD4, Caltag Laboratories, Burlingame, CA, USA). The frozen section was washed with Tris HCl buffer (pH, 7.6) and was reacted with the first antibody overnight. It was then reacted with alkaline phosphatase-labeled anti-rat IgG mouse monoclonal antibody (Dako Cytomation, Kyoto, Japan) for 4 h and with APAAP complex (Dako Cytomation) for 4 h. The reaction products were visualized using a Fast Red matrix kit (Dako Cytomation). Counterstaining was performed with hematoxylin stain.

After the staining, photographs were taken with a light microscope (BH2, Olympus, Tokyo, Japan). Digital images

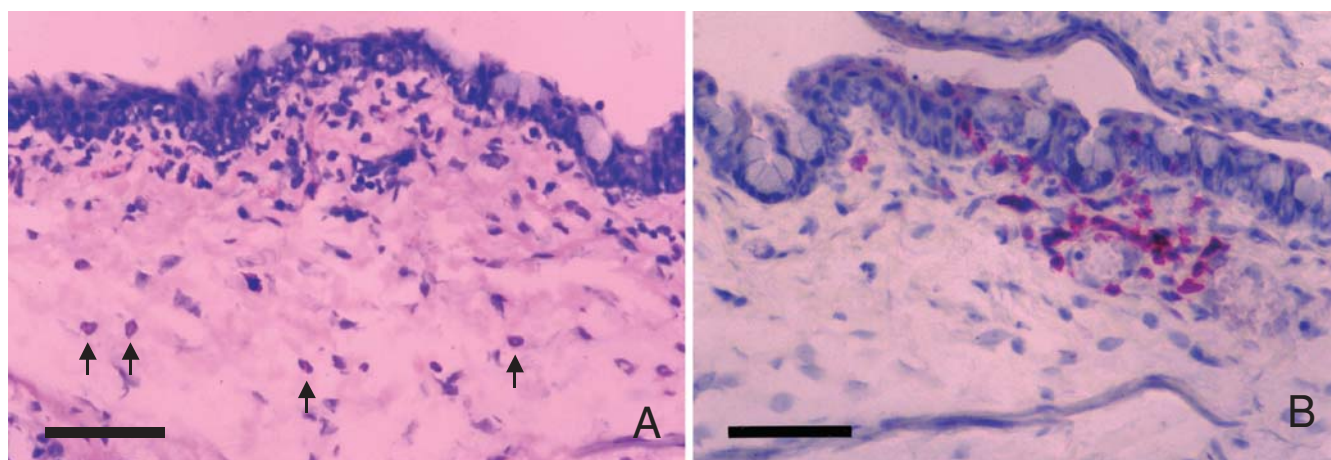


Figure 2A,B. Histopathology and immunohistochemistry of conjunctival tissues in the allergy group. **A** eosinophils (*arrows*) (acid Giemsa staining). Bar = 50 μm. **B** CD4-positive cells [positive cells stained red by the alkaline phosphatase–antialkaline phosphatase (APAAP) method.] Bar = 50 μm.

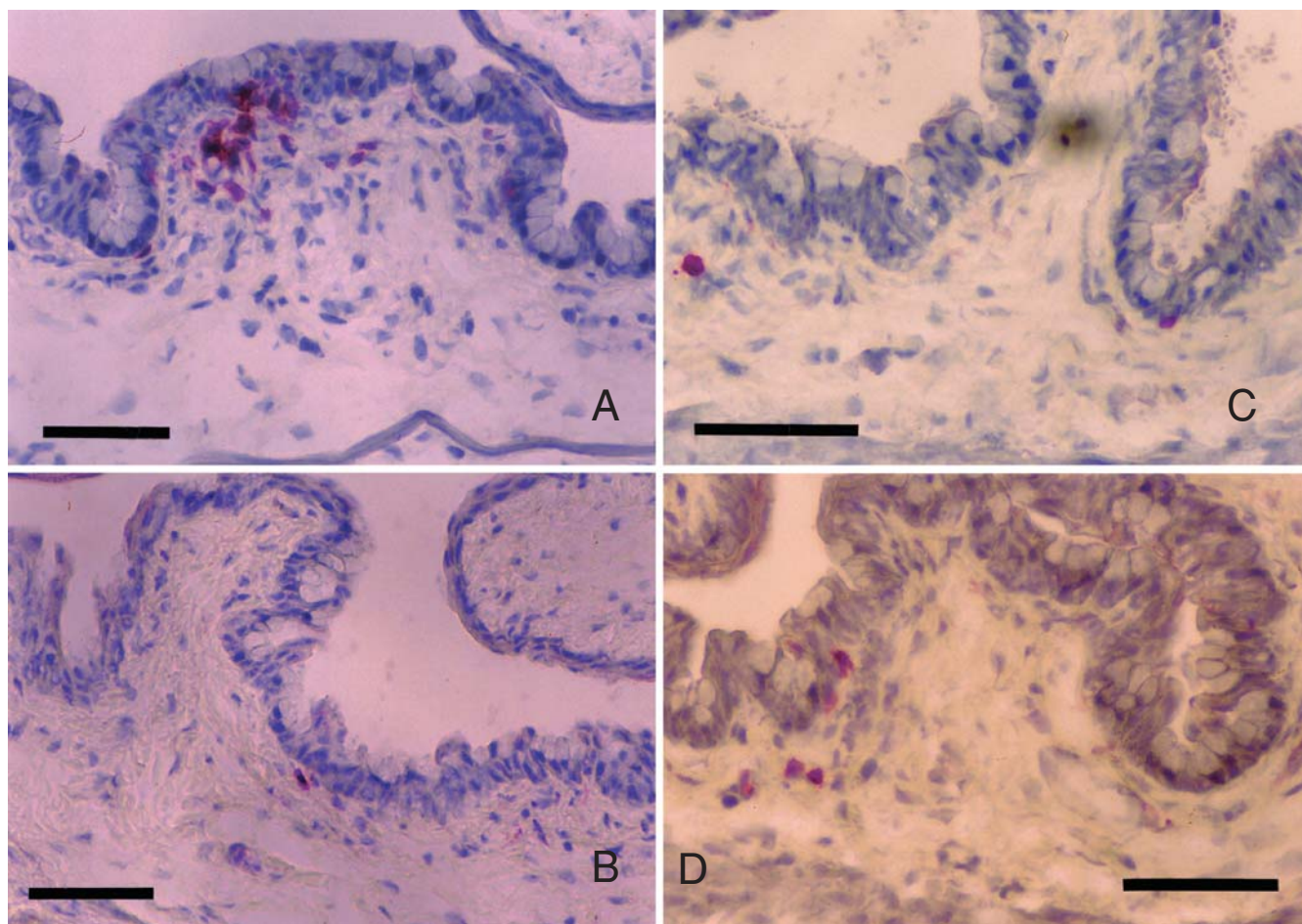


Figure 3A–D. CD4-positive cells in conjunctival tissues. **A** allergy group; **B** Dx group; **C** FK group; **D** negative control group. CD4-positive cells are stained red by the APAAP method. Bar = 50 μm.

were processed by a film scanner, and the total area of the subconjunctival tissue in the digital image was calculated using a semiautomated image analysis method on a computer (Apple Macintosh) with Photoshop 6.0 software (Adobe Systems, San Jose, CA, USA). The density per square millimeter of eosinophils stained with acid Giemsa staining and of CD4-positive cells stained red by APAAP in the subconjunctival tissue was counted. The cell densities of 15 thin sections for each specimen were counted. Statistical analysis was performed by the Mann-Whitney *U* test, and the difference was regarded as statistically significant at the less than 5% level.

Reverse Transcriptase Polymerase Chain Reaction Method

Removed conjunctival tissues including lacrimal glands were preserved in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) at -80°C . The conjunctival tissues were homogenized with a homogenizer (VP-55, Taitec, Saitama, Japan), and total RNA was extracted using the RNeasy mini kit (Qiagen).

The primer used in the analysis of productive *C* ϵ gene was 5'-TGGACTACTGGGGTCAAGG-3' (forward) and 5'-AGCGATGAATGGAGTAGC-3' (reverse),¹⁰ and a one-step reverse transcriptase polymerase chain reaction (RT-PCR) of the productive *C* ϵ gene was performed with the Qiagen one-step RT-PCR kit. The 50- μl RT-PCR amplification contained the following final components: 5 μl aliquot of total RNA, 10 μl of 5 $\times$ buffer, 400 μM of deoxyribonucleotide triphosphate, 0.5 μM of sense and antisense primers, and 2.0 μl of enzyme mix. One-step RT-PCR was performed by reverse transcription at 50°C for 30 min, initial PCR activation at 95°C for 20 min, and PCR for 30 cycles in a thermal cycler (Geneamp PCR system 9700, Applied Biosystems Japan, Tokyo, Japan). Each PCR cycle contained a denaturation step at 94°C for 5 min, an annealing step at 60°C for 5 min, followed by 30 cycles of 1.5 min at 72°C , 1.5 min at 94°C , and 1.5 min at 60°C , with final extension at 72°C for 10 min. For analysis of RT-PCR products, electrophoresis was performed with 1.5% agarose gel, and the resultant products were visualized with ethidium bromide to reveal the DNA. RT-PCR was also performed on hypoxanthine/guanine phosphoribosyltransferase (HGPRT) as an internal control.

Results

Histological Study

In the allergy group, infiltration of eosinophils was observed in the subconjunctival tissue by acid Giemsa staining (Fig. 2A), and infiltration of CD4-positive cells was observed around the blood vessels and subepithelial area in subconjunctival tissues by immunohistochemical methods (Fig. 2B). In the Dx group (Fig. 3B) and the FK group (Fig. 3C),

infiltration of eosinophils and CD4-positive cells was less than in the allergy group (Fig. 3A).

The cell density of eosinophils infiltrating into subconjunctival tissues was 66.3 ± 34.7 cells/ mm^2 (mean $\pm$ SD) for the allergy group; 2.5 ± 4.2 for the Dx group; 4.2 ± 8.6 for the FK group; and 3.7 ± 7.6 for the negative control group (Fig. 4). The cell density of CD4-positive cells infiltrating into subconjunctival tissues was 145.9 ± 66.4 for the allergy group; 10.0 ± 12.9 for the Dx group; 10.1 ± 15.5 for the FK group; and 21.7 ± 17.8 for the negative control group (Fig. 5). The allergy group demonstrated significant differences in eosinophil and CD4-positive cell counts compared with the Dx group ($P < 0.0001$), the FK group ($P < 0.0001$), and the negative control group ($P < 0.0001$) (Figs. 4, 5).

Productive *C* ϵ Gene

The expression of productive *C* ϵ gene was positive only in the Dx group, but not in the allergy group, the FK group, or the negative control group (Fig. 6). A positive ratio of productive *C* ϵ gene expression in the Dx group was obtained in three of the six experimental allergic conjunctivitis model mice in this group.

Discussion

Employing the mouse experimental allergic conjunctivitis model generated by sensitizing Balb/c mice with ovalbumin (OVA), we studied allergic inflammation histologically in the conjunctival tissues. Eosinophil and CD4-positive T-cell infiltrations were observed in conjunctival tissues in the

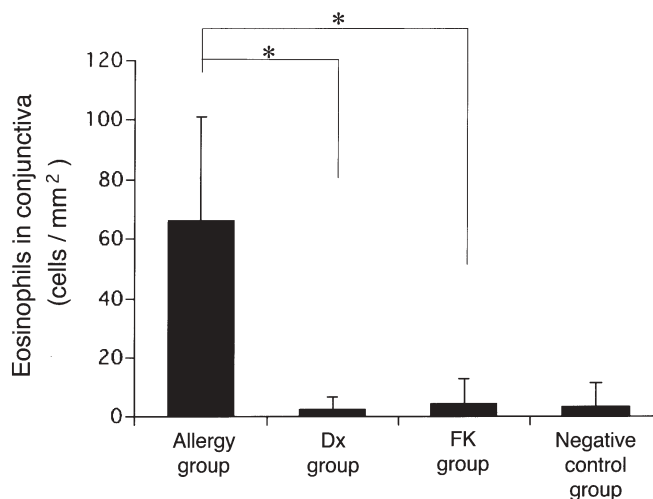


Figure 4. Effects of dexamethasone and FK506 ointment on eosinophil infiltration in the conjunctival tissues. Values are mean $\pm$ SD. Significant differences were found between the allergy group and the Dx group ($P < 0.0001$), and between the allergy group and the FK group ($P < 0.0001$). *Mann-Whitney *U* test, $P < 0.0001$.

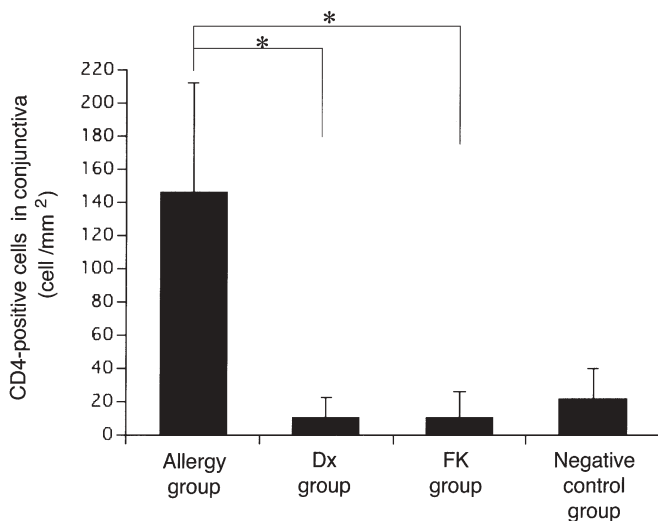


Figure 5. Effects of dexamethasone and FK506 ointment on CD4-positive cell infiltration in the conjunctival tissues. Values are mean $\pm$ SD. Significant differences were found between the allergy group and the Dx group ($P < 0.0001$), and between the allergy group and the FK group ($P < 0.0001$). *Mann-Whitney U test, $P < 0.0001$.

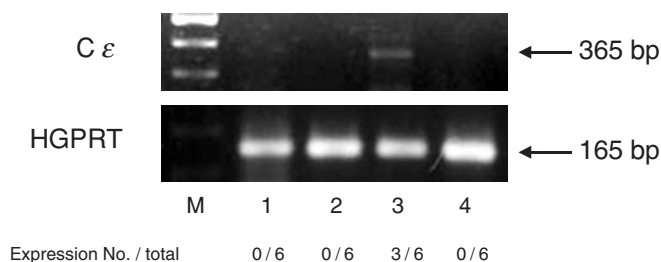


Figure 6. Effects of dexamethasone and FK506 ointment on productive $C\epsilon$ gene expression in the conjunctival tissues including the lacrimal gland. M, marker; lane 1, negative control group; lane 2, FK group; lane 3, Dx group; lane 4, allergic group. $C\epsilon$, productive $C\epsilon$ gene; HGPRT, hypoxanthine/guanine phosphoribosyltransferase.

allergy group of our mouse experimental allergic conjunctivitis model, the same as in previous reports^{11,12} on allergic inflammation in conjunctiva. Furthermore, we examined the efficacy of topical dexamethasone and FK506 instillations to suppress allergic inflammation of the conjunctiva in the mouse experimental allergic conjunctivitis models. Both topical treatments suppressed eosinophil infiltration and CD4-positive T-cell infiltration into conjunctival tissues, showing no remarkable difference in the degree of suppression of cell infiltration between the two drugs. Iwamoto et al.⁸ studied the effect of systemic administration and instillation of FK506 on a rat experimental allergic blepharoconjunctivitis model. They reported that FK506 suppressed allergic blepharoconjunctivitis in a dose-dependent manner and that the effect of instillation on cell infiltration into the conjunctiva was weaker than by systemic adminis-

tration. Sengoku et al.¹³ reported that FK506 eyedrops inhibit late and delayed-type responses in several animal models of ocular allergy.¹³ In this study, both topical steroid and FK506 instillations were effective on the allergic inflammation in the mouse allergic conjunctivitis model. The efficacy of topical FK506 ointment on atopic dermatitis has been reported previously.^{14,15} These reports and our results suggest that topical administration of FK506 as eyedrops or ointment is also useful for treating allergic conjunctival disease.

The $C\epsilon$ gene is expressed as a result of the process of a B cell changing into an IgE-producing plasma cell. The germline $C\epsilon$ gene is first expressed in B cells, and finally, the productive $C\epsilon$ gene is expressed in plasma cells. Durham et al.¹⁶ conducted a provocation study in patients with hay fever and detected expression of the $C\epsilon$ gene in nasal mucosa. They reported that the expression of the $C\epsilon$ gene increased after provocation and was suppressed by local administration of a corticosteroid (fluticasone propionate). Nagai et al.¹⁰ studied $C\epsilon$ gene expression in the cervical lymph node using a contact dermatitis mouse model. They reported that neither FK-506 nor cyclosporin A suppressed $C\epsilon$ gene expression. In this study, the expression of the productive $C\epsilon$ gene in conjunctival tissues, including the lacrimal gland, was examined. Expression of the productive $C\epsilon$ gene was not detected in the negative control, the allergy, or the FK groups, and was positive only in the Dx group.

Regarding the effect of corticosteroids on IgE production, various reports have been published. Hiratsuka et al.¹⁷ studied the IgE antibody level in the supernatant of cultivated B cells from the peripheral blood of patients with atopic dermatitis after local administration of steroid ointment. They reported that the IgE antibody increased in only the steroid administration group and that IgG, IgA, and IgM remained unchanged. Their in vitro study suggested that steroid treatment for allergic diseases increases the production of IgE antibody. On the other hand, Benson et al.¹⁸ reported that topical corticosteroid treatment in patients with allergic rhinitis decreased IgE, Th2 cytokines, eosinophil cationic protein, and eosinophils in the nasal fluid. These findings suggest that corticosteroids reduce IgE antibody levels in vivo. The discrepancy in the results between in vitro and in vivo studies is difficult to explain, and the efficacy of corticosteroids on IgE production still remains unclear. In addition, an increase in serum IgE levels, as a paradoxical effect, has also been reported in a case of X-linked autoimmune enteropathy treated by systemic administration of cyclosporin A and FK506.¹⁹

These reports indicate that the efficacy of corticosteroids and immunosuppressants on $C\epsilon$ gene expression and IgE antibody production are due to different mechanisms, depending on the drug concentrations, drug administration methods, and target tissues or target cells in, for example, local lymph nodes or mucosal tissues. Regarding topical dexamethasone instillation in the present study, the expression of the productive $C\epsilon$ gene was found in the conjunctival tissues, including the lacrimal gland, in the case of the

Dx group but not in the normal conjunctiva (negative control group). Thus, it is suggested that topical corticosteroid instillation may increase the local production of IgE antibody in conjunctiva. In contrast, the present results showed that FK-506 instillation had no effect on the local production of IgE antibody. However, a controlled trial of different doses of corticosteroids and FK506 would be necessary to determine the effects on local IgE production.

In conclusion, although topical steroid and topical FK506 instillations are both effective in the suppression of eosinophil and lymphocyte infiltration, different therapeutic mechanisms and efficacy on local IgE production should be considered for these two drugs. The production of IgE and IgG4 was selectively enhanced during the rebound phenomenon after steroid discontinuation.²⁰ It may be especially important to study the rebound phenomenon of these two drugs in patients with ACD.

References

1. Tuft SL, Cree IA, Woods M, Yorston D. Limbal vernal keratoconjunctivitis in the tropics. *Ophthalmology* 1998;105:1489–1493.
2. BenEzra D, Pe'er J, Brodsky M, Cohen E. Cyclosporine eyedrops for the treatment of severe vernal keratoconjunctivitis. *Am J Ophthalmol* 1986;101:278–282.
3. Secchi AG, Tognon MS, Leonardi A. Topical use of cyclosporine in the treatment of vernal keratoconjunctivitis. *Am J Ophthalmol* 1990;110:641–645.
4. Bleik JH, Khafid F, Tabbara F. Topical cyclosporine in vernal keratoconjunctivitis. *Ophthalmology* 1991;98:1679–1684.
5. Andersson J, Nagy S, Groth CG, Andersson U. Effects of FK506 and cyclosporine A on cytokine production studied in vitro at a single-cell level. *Immunology* 1992;75:136–142.
6. Wang SC, Morel PA, Wang Q, Jordan ML, Simmons RL, Twardy DJ. A dual mechanism of immunosuppression by FK-506. *Transplantation* 1993;56:978–985.
7. de Paulis A, Crillo R, Ciccarelli A, de Crescenzo G, Oriente A, Marone G. Characterization of the anti-inflammatory effect of FK-506 on human mast cells. *J Immunol* 1991;147:4278–4285.
8. Iwamoto H, Yoshida H, Yoshida O, Fukushima A, Ueno H. Inhibitory effects of FK506 on the development of experimental allergic/immune-mediated blepharoconjunctivitis in Lewis rats by systemic but not by topical administration. *Graefes Arch Clin Exp Ophthalmol* 1999;237:407–414.
9. Rikkers SM, Holland GN, Drayton GE, Michel FK, Torres MF, Takahashi S. Topical tacrolimus treatment of atopic eyelid disease. *Am J Ophthalmol* 2003;135:297–302.
10. Nagai H, Hiyama H, Matsuo A, Ueda Y, Inagaki N, Kawada K. FK-506 and cyclosporine A potentiate the IgE antibody production by contact sensitization with hapten in mice. *J Pharmacol Exp Ther* 1997;283:321–327.
11. Magone MT, Chan CC, Rizzo LV, Kozhich AT, Whitcup SM. A novel murine model of allergic conjunctivitis. *Clin Immunol Immunopathol* 1998;87:75–84.
12. Merayo-Lloves J, Zhao TZ, Dutt JE, Foster CS. A new murine model of allergic conjunctivitis and effectiveness of nedocromil sodium. *J Allergy Clin Immunol* 1996;97:1129–1140.
13. Sengoku T, Sakuma S, Satoh S, et al. Effect of FK506 eye drops on late and delayed-type responses in ocular allergy models. *Clin Exp Allergy* 2003;33:1555–1560.
14. Ruzicka T, Bieber T, Schopf E, et al. A short-term trial of tacrolimus ointment for atopic dermatitis. European Tacrolimus Multicenter Atopic Dermatitis Study Group. *N Engl J Med* 1997;337:816–821.
15. Hanifin JM, Ling MR, Langley R, Breneman D, Rafal E. Tacrolimus ointment for the treatment of atopic dermatitis in adult patients: part I, efficacy. *J Am Acad Dermatol* 2001;44:S28–S38.
16. Durham SR, Gould HJ, Thienes CP, et al. Expression of ϵ germ-line gene transcripts and mRNA for the ϵ heavy chain of IgE in nasal B cells and the effects of topical corticosteroid. *Eur J Immunol* 1997;27:2899–2906.
17. Hiratsuka S, Yoshida A, Ishioka C, Kimata H. Enhancement of in vitro spontaneous IgE production by topical steroids in patients with atopic dermatitis. *J Allergy Clin Immunol* 1996;98:107–113.
18. Benson M, Strannegard IL, Strannegard O, Wennergren G. Topical steroid treatment of allergic rhinitis decreases nasal fluid TH2 cytokines, eosinophils, eosinophil cationic protein, and IgE but has no significant effect on IFN- γ , IL-1 β , or neutrophils. *J Allergy Clin Immunol* 2000;106:307–312.
19. Kawamura N, Furuta H, Tame A, et al. Extremely high serum level of IgE during immunosuppressive therapy: paradoxical effect of cyclosporine A and tacrolimus. *Int Arch Allergy Immunol* 1997;112:422–424.
20. Kimata H. Selective enhancement of production of IgE, IgG4, and Th2-cell cytokine during the rebound phenomenon in atopic dermatitis and prevention by suplatast tosylate. *Ann Allergy Asthma Immunol* 1999;82:293–295.