

Transconjunctival immunotherapy using cholera toxin B to treat experimental allergic conjunctivitis in a mouse model

Aki Oikawa · Jun Shoji · Noriko Inada ·
Mitsuru Sawa

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Abstract

Purpose We evaluated the use of immunological biomarkers in transconjunctival immunotherapy by using cholera toxin B for treating experimental allergic conjunctivitis in a mouse model.

Methods Balb/c mice were sensitized using intraperitoneal injections of ovalbumin (OVA) and were then divided into two groups. The first group was treated by topical instillation of OVA after the instillation of combined OVA and cholera toxin B (CTB) solution B group). The second group was treated by topical instillation of OVA alone (allergy group). The control group consisted of nonsensitized mice undergoing topical OVA instillation only. The numbers of eosinophils and CD4-positive lymphocytes in the conjunctiva were determined histologically, and the observation of immunoglobulin A (IgA)-positive cells in the conjunctiva was performed by immunohistochemistry. Cytokine concentration in the conjunctiva was determined by the protein-array methods. Messenger RNA expression of T-cell-specific markers, such as T-bet, GATA-3, and Foxp3, in the conjunctiva was detected by reversed transcriptase polymerase chain reaction.

Results The number of eosinophils and CD4-positive lymphocytes increased significantly in the allergy group compared with the control group ($P < 0.001$) but showed no difference between the CTB group and control group. Concentrations of interleukin 4 (IL-4) ($P < 0.05$), B-lymphocyte chemoattractant ($P < 0.01$), and thymus-expressed

chemokine ($P < 0.05$) in the conjunctiva were significantly higher in the CTB group than in the other two groups. GATA-3 messenger RNA (mRNA) in the conjunctiva was expressed in the three groups, but T-bet and Foxp3 were not detected.

Conclusion Transconjunctival immunotherapy using CTB can be evaluated by histological examination of eosinophils and CD4-positive T cells, and a mucosal immunity-associated chemokine and a helper T-cell-17-associated chemokine as biomarkers.

Keywords Experimental allergic conjunctivitis · Chemokine · Cholera toxin B · Eosinophil · Immunotherapy

Introduction

Allergen-specific immunotherapy (SIT) for treating allergic diseases is a therapeutic method aiming to achieve hypsensitization by administrating increasing doses of allergens. SIT involves the administration of allergen extracts using a standardized regimen, usually subcutaneously [subcutaneous allergen immunotherapy (SCIT)], or, increasingly, sublingual allergen immunotherapy (SLIT) [1]. For nearly a century, SIT has motivated the development of newer, alternative methods of allergen administration for improving treatment and compliance effects and decreasing adverse events.

We reported the efficacy of cholera toxin B (CTB) as an adjuvant during transconjunctival immunotherapy for experimental allergic conjunctivitis (EAC) in guinea pigs. We concluded that transconjunctival immunotherapy using CTB could suppress clinical effects, eosinophil infiltration into the conjunctival epithelium, and conjunctival

A. Oikawa (✉) · J. Shoji · N. Inada · M. Sawa
Division of Visual Sciences, Department of Ophthalmology,
Nihon University School of Medicine,
30-1 Oyaguchi-Kamimachi, Itabashi-ku,
Tokyo 173-8610, Japan
e-mail: aoikawa@med.nihon-u.ac.jp

epitheliopathy [2]. However, the guinea pig is an unsuitable experimental animal for elucidating an immunologic mechanism of transconjunctival immune therapy. In this study, we investigated chemokines associated with transconjunctival immune therapy using a mouse model.

The aim of the investigation was to determine a clinically applicable biomarker to evaluate the efficacy of transconjunctival immunotherapy in mice with EAC, which was induced in mice sensitized with ovalbumin (OVA). We evaluated infiltrated inflammatory cells, cytokine and chemokine expression, and T cell-specific molecular markers in the conjunctiva of the mouse model.

Materials and methods

Experimental allergic conjunctivitis

Thirty-one Balb/c mice was used in this study. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Resolution Regarding Animals used in Research. Mice were divided into three groups: allergy group (ten eyes of ten mice: 10/10), CTB group (11/11), and control group (10/10), as described below.

Mice in the allergy and CTB groups were sensitized by a previously reported methods [3] (Fig. 1). On days 1 and 8, 0.2 ml intraperitoneal injections of a homogenized solution of ovalbumin (OVA: Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 50 µg/ml and hydroxyl-aluminum gel (Cosmobio, Tokyo, Japan) at a concentration of 150 mg/ml in saline was carried out (sensitization methods). Both the allergy and CTB groups underwent topical instillation of OVA solution (5 mg/ml) of 25 µl *oculus uterque* (OU) on days 15 and 17 (antigen challenge method). In the CTB groups, a combined solution of CTB (List Biological Laboratories, Campbell, CA, USA) (8 µg/30 ml) and OVA (20 µg/30 ml) of 25 µl was given on days 8 and 15. In the control group, mice underwent topical instillation of OVA solution on days 15 and 17 only (treatment by instillation immunotherapy).

On day 17, at 12 h after the last instillation of the OVA solution, all mice were euthanized by intraperitoneal injection of an overdose of pentobarbital (Nembutal, Abbott Laboratories, Abbott Park, IL, USA), and their eyelids and globes were sampled. One eye was used for histological examination, and the palpebral and bulbar conjunctiva of the fellow eye was used to examine cytokine and chemokine profiles and T-bet, GATA-3, and Foxp3 mRNA expression, the specific markers of T-helper-cell type 1 (Th1), Th2, and regulatory T cell (Treg), respectively.

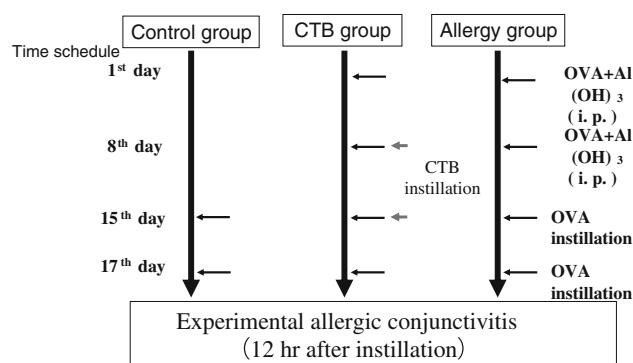


Fig. 1 Protocol of instillation immunotherapy in experimental allergic conjunctivitis. Homogenized combined solution of ovalbumin (OVA, 50 µg/ml) and hydroxy aluminum [Al (OH)₃, 150 mg/ml] were administered to Balb/c mice intraperitoneally (0.2 ml) for sensitization. Either OVA (5 mg/ml) or cholera toxin B solution (CTB) solution [combined solution of OVA (20 µg/30 ml) and CTB (8 µg/30 ml)] was instilled topically *oculus uterque* (OU) following the experimental protocol shown in this figure

Histological studies

Frozen sections were made for histological study, and the number of infiltrated eosinophils and CD4-positive lymphocytes in subconjunctival tissue were counted, as explained below. Distribution of IgA-positive cells in the conjunctival tissues was investigated histologically.

The sampled eyelids and globes were fixed immediately using 4% periodate–lysine–paraformaldehyde (PLP) solution then embedded using optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Seiki, Tokyo, Japan), and frozen by the dry-ice-isopentane method. Using a cryostat (HM505E, Microm, Walldorf, Germany), 8-µm-thick frozen sections were made and stained with acid Giemsa stain to count the eosinophils using Diff-Quick® (Sysmex, Kobe, Japan). To count CD4-positive lymphocytes, frozen sections were treated by the alkaline phosphatase antialkaline phosphatase (APAAP) method in which anti-mouse CD4 rat monoclonal antibody (Clone; CT-CD4, Caltag Laboratories, Burlingame, CA, USA) was used as the first antibody. The immunohistochemical observation of IgA-positive cells in the conjunctival tissues was performed using the APAAP method, which uses anti-mouse IgA rat monoclonal antibody (Clone: C10-1, BD Pharmingen, NJ, USA) as the first antibody.

Stained specimens were observed using a light microscope (BH2, Olympus, Tokyo, Japan), and cell densities (cells/mm²) of eosinophils and CD4-positive lymphocytes in the subconjunctival tissue were determined using the image analysis system.

Cytokine and chemokine profiles in conjunctival tissue

Resected conjunctival tissue was preserved in Eagle's minimum essential medium (MEM) α -modification medium (Sigma–Aldrich) at room temperature for 6 h. Then, following the product information, the protein-array method, RayBio Mouse Inflammation Antibody Array I (Ray Biotech, Norcross, GA, USA) was applied using the culture medium. The membranes were blocked with a blocking buffer, and 1 ml of culture medium was added, followed by incubation at 4°C over night. Membranes were washed, and 1 ml of primary biotin-conjugated antibody was added, followed by incubation at room temperature for 2 h. Next, membranes were incubated in 2 ml of horseradish-peroxidase-conjugated streptavidin at room temperature for 30 min. Then, membranes were developed using an enhanced chemiluminescence-type solution. After the membranes were exposed to BioMax Light Film (Kodak Japan, Tokyo, Japan), they were processed. The optical density (OD: Odu/mm²) was determined by densitometry using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA). Protein concentration in the culture medium was determined using the bicinchoninic acid (BCA) method (BCATM Protein Assay Kit, Pierce, Rockford, IL, USA), and the optical density (OD) value obtained by the protein-array method was calibrated.

T-bet, GATA-3, and Foxp3 mRNA expression in conjunctival tissue

Total RNA (tRNA) was extracted from the removed conjunctival tissues after homogenization using the RNeasy® MiniKit (Qiagen, Hilden, Germany). Then, gene expressions of T-bet, GATA-3, and Foxp3 were examined by the reverse transcriptase polymerase chain reaction (RT-PCR). Specific pairs of primers and length of transcripts for T-bet, GATA-3, and Foxp3 are shown in Table 1. RT-PCR was carried out using the Qiagen One-Step RT-PCR Kit with thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA) following product instructions. For Foxp3 mRNA detection, RNA was reverse transcribed for 30 min at 50°C, and the reverse transcriptase was inactivated by 15-min incubation at 95°C. The amplification profile was 32 cycles of 93°C for 30 s, 60°C for 30 s, and 72°C for 60 s, followed by a 7-min extension at 72°C. For GATA-3 mRNA detection, the

amplification profile was 32 cycles of 93°C for 30 s, 56°C for 30 s, and 72°C for 60 s. For T-bet mRNA detection, the amplification profile was 35 cycles of 93°C for 30 s, 60°C for 60 s, and 72°C for 60 s. For RT-PCR product analysis, 1% agarose gel electrophoresis (AGE) was done.

Statistical analysis

Cell density of eosinophils and CD4-positive lymphocytes in the subconjunctiva among the three groups of mice was analyzed by the Kruskal–Wallis H test. OD values in the protein-array experiment were analyzed by one-way analysis of variance (ANOVA). Any difference <5% level was regarded as statistically significant.

Results

Histological studies

Cell density (cells/mm²) of infiltrated eosinophils and CD4-positive lymphocytes in the subconjunctiva are shown in Tables 2 and 3. The allergy group showed significantly higher cell density in both eosinophils and CD4-positive lymphocytes compared with the control group (eosinophil $P < 0.001$ and CD4-positive lymphocytes $P < 0.001$, respectively) and the CTB group (eosinophil $P < 0.001$ and CD4-positive lymphocytes $P < 0.05$, respectively). However, there was no significant difference in either cell densities between the CTB and control groups (Tables 2, 3; Fig. 2).

Regarding IgA-positive cells in subconjunctival tissue, there were no differences among the three groups. In

Table 2 Histological analysis of eosinophils in conjunctiva

Group	Eosinophils in conjunctiva (Mean $\pm$ SD) (Cells/mm ²)	P value
Control	88.1 $\pm$ 65.7	$\left. \begin{array}{l} \text{NS} \\ P < 0.001 \end{array} \right\} P < 0.001$
CTB	142.1 $\pm$ 103.7	
Allergy	428.9 $\pm$ 354.2	

CTB cholera toxin B, NS not significant

Table 1 Primers to detect Foxp3, GATA-3, and T-bet messenger RNA

	Forward	Reverse
Foxp3	3'-GGCGAAAGTGGCAGAGAGGTAT-5'	3'-AAGACCCAGTGGCAGCAGAA-5'
GATA-3	3'-CTGGAGGAGGAACGCTAATG-5'	3'-GGTTGAAGGAGCTGCTCTTG-5'
T-bet	3'-TGCCCCGCTTCTCTCCAACCAA-5'	3'-TGCCTGCAGTGCTTCTAACA-5'

conjunctival tissue, lymphoid accumulation equivalent to conjunctiva-associated lymphoid tissue (CALT) was found, and CALT was distinguished histologically in the follicular and parafollicular areas. The parafollicular area in the CALT of the CTB group including a large number of IgA-positive cells was enlarged compared with that of the control and allergy groups (Fig. 3).

Cytokine and chemokine profiles in conjunctival tissue

Interferon-gamma (IFN- γ) and interleukin (IL)-2 in the Th1 cytokine group were at a low level in all three groups, and there was no significant difference among groups. IL-4 and IL-13 in the Th2 of the cytokine group increased in both the allergy and the CTB groups compared with the control group. IL-4, in particular, increased significantly in the CTB group ($P < 0.05$) (Fig. 4).

Table 3 Immunohistochemical analysis CD4-positive lymphocytes in conjunctiva

Group	CD4 positive lymphocytes in conjunctiva (Mean $\pm$ SD) (Cells/mm ²)	P value
Control	307.2 $\pm$ 131.5	$\left. \begin{array}{l} \text{NS} \\ P < 0.05 \end{array} \right\} P < 0.001$
CTB	419.9 $\pm$ 105.5	
Allergy	880.3 $\pm$ 257.2	

CTB cholera toxin B, NS not significant

One chemokine, eotaxin, which has a chemoattractive effect on eosinophils and T-cell-activation gene-3 (TCA-3), inducing Th2-related substances, showed the same levels of increase in both the allergy and CTB groups. However, the increase of this chemokine was not statistically significantly different from controls. A monokine induced by gamma interferon (MIG) and interferon-inducible T-cell alpha chemoattractant (I-TAC), which induces Th1, showed no differences in the three groups of mice. B-lymphocyte chemoattractant (BLC) ($P < 0.01$) and thymus-expressed chemokine (TECK) ($P < 0.05$), which induces the homing phenomenon of B cells, showed a significant increase in the CTB group compared with the control group (Fig. 5).

T-bet, Foxp3, GATA-3 mRNA expression in the conjunctiva

GATA-3 mRNA was expressed in the conjunctiva of all three groups. However, T-bet mRNA and Foxp3 mRNA expression was not found in any group (Fig. 6).

Discussion

SCIT is the main clinically applicable allergen-specific immunotherapy. It was first reported by Cohen et al. in 1911 [4]. They applied gradual administration of increasing doses of the specific causative allergen in patients with hay fever. The recently developed methods of SIT can be classified into two types: that using a substitutional

Fig. 2 Immunohistochemical localization of CD4-positive T cells in conjunctiva. CD4-positive lymphocytes stained red by immunohistochemistry are observed in the epithelial layer (arrowhead) and subconjunctival tissue (arrow). In the allergy group, a marked infiltration of CD4-positive lymphocytes is observed in the subconjunctival tissue.
a Control group; **b** cholera toxin B solution (CTB) group, and **c** allergy group. Bar 50 μ m

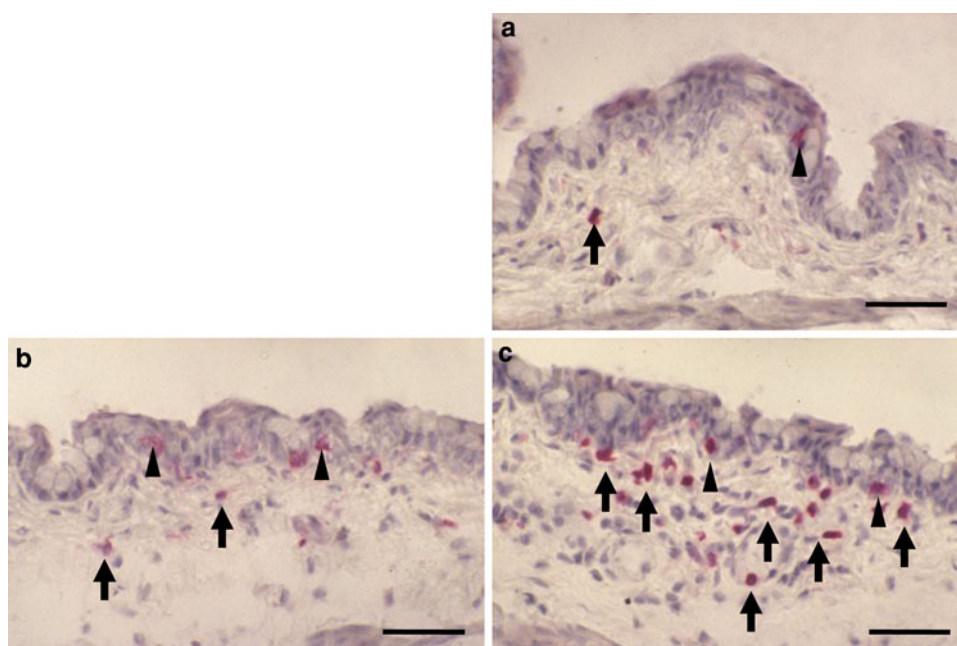


Fig. 3 Immunoglobulin A (IgA)-positive cells in the conjunctiva. Conjunctiva-associated lymphoid tissue is divided into follicular area (FA) and parafollicular area (PFA) histologically. PFA in conjunctiva-associated lymphoid tissue (CALT) of the cholera toxin B solution (CTB) group including a large number of IgA-positive cells (*arrow*) is enlarged compared with that of the control and allergy groups (immunohistochemistry for IgA): **a** control group; **b** CTB group; **c** allergy group. Bar 50 μ m

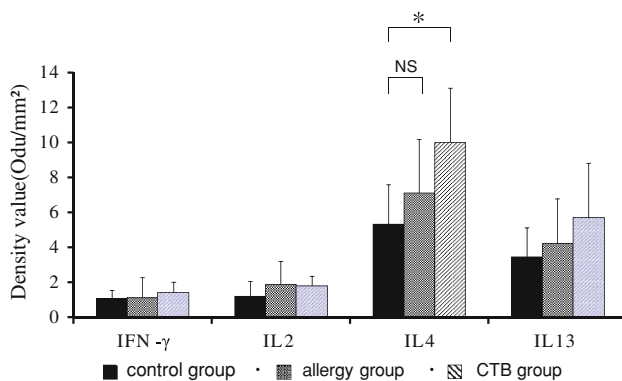
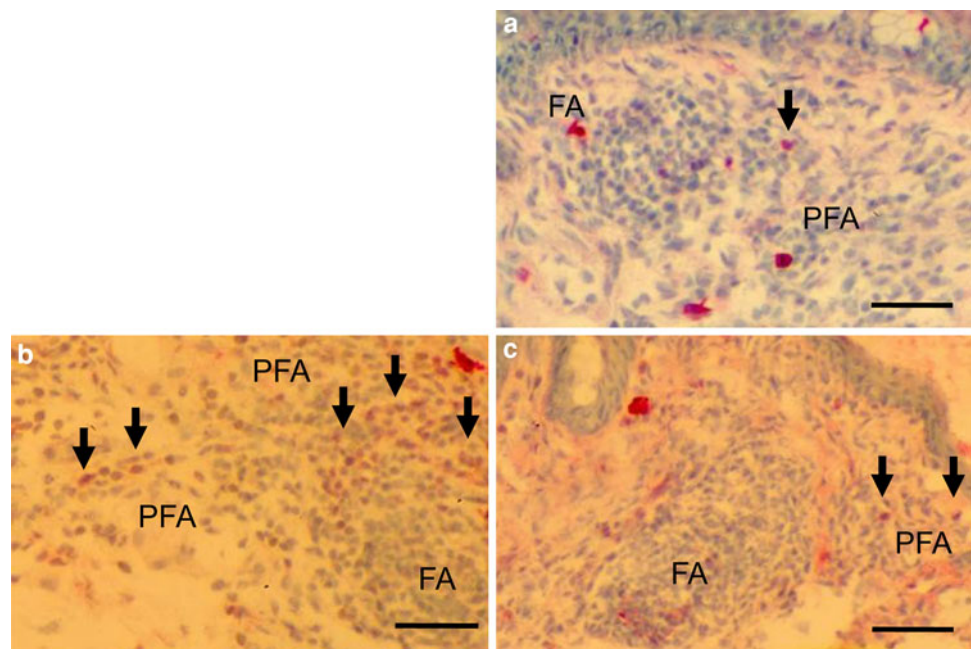


Fig. 4 Cytokine profile in conjunctival tissue. Interleukin-4 (IL-4) in the cholera toxin B (CTB) group shows a significant increase over that in the control group ($P < 0.05$). $*P < 0.05$, analysis of variance (ANOVA). *NS* not significant, *IFN- γ* interferon gamma, *IL* interleukin

alternation of an antigen, such as in the DNA vaccine method in which plasmid DNA is used instead of protein [5] and changing the administration route of the specific antigen, such as in oral administration, in treating allergic conjunctivitis patients [6]; or that local sublingual immunotherapy when treating allergic conjunctivitis patients [7]. Both are reported to induce immunological tolerance. We reported the efficacy of transconjunctival immunotherapy using CTB as an adjuvant for EAC in guinea pigs [2]. In those studies, improvement in clinical scores and eosinophil suppression in conjunctival tissue were achieved in the treated group. However, a clinically applicable tool has not been established to evaluate the efficacy of this immunotherapy. In sublingual immunotherapy for seasonal allergic rhinitis, eosinophil cationic protein (ECP) in nasal

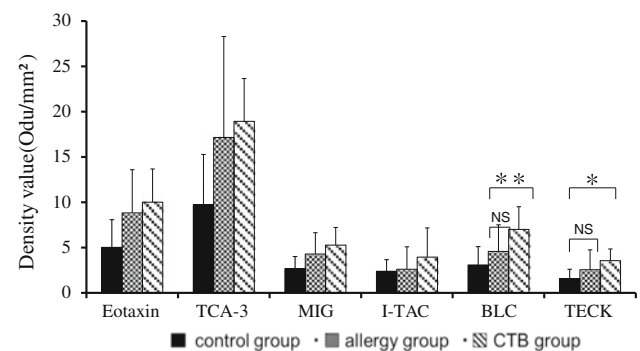


Fig. 5 Chemokine profile in conjunctival tissue. B-lymphocyte chemoattractant (BLC) and thymus-expressed chemokine (TECK) in the cholera toxin B (CTB) group show a significant increase over those in the control group. $*P < 0.05$, analysis of variance (ANOVA), $**P < 0.01$, ANOVA; *NS* not significant, *TCA-3*, T-cell activation gene 3, *MIG* monokine-induced by gamma interferon, *I-TAC* interferon-inducible T-cell alpha chemoattractant

discharge or serum has been reported to be a useful index [8]. It is necessary to establish an evaluation method for the therapeutic effects of transconjunctival immunotherapy. Therefore, in this study, we sought a clinically applicable biomarker to evaluate the efficacy of topical immunotherapy using CTB in treating EAC in a mouse model.

Adjuvants play an important role in immunotherapy by facilitating the induction of immunological tolerance. CTB has the potential of becoming an effective adjuvant. Combined administration of an antigen with CTB in oral immunotherapy was effective in suppressing allergic reaction, not only by suppressing the newly produced antigen-specific IgE [9], but also by suppressing reaction by the preformed

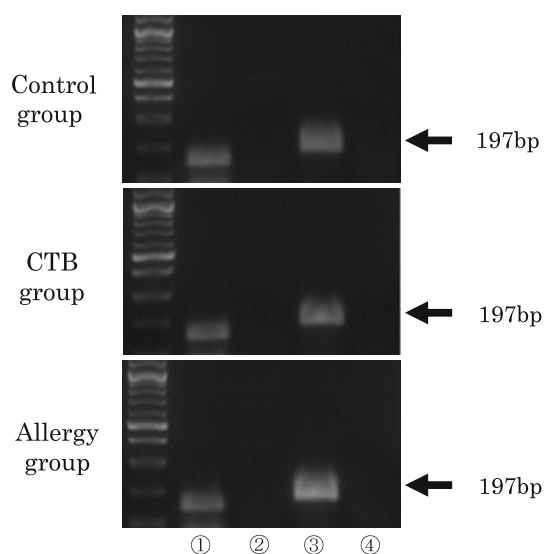


Fig. 6 GATA-3, Foxp3, T-bet messenger RNA (mRNA) in conjunctival tissue. GATA-3 expression is positive in all three groups. However, Foxp3 and T-bet mRNA are both negative in all groups. There is no difference in mRNA expression among groups. Lane 1, hypoxanthine/guanine phosphoribosyltransferase (HGPRT; 190 bp), lane 2 Foxp3 (600 bp), lane 3 GATA-3 (197 bp), lane 4 T-bet (900 bp)

antigen-specific antibody and suppressing the Th1-dependent delayed-type hypersensitivity [9]. Furthermore, after sublingual administration of an antigen with CTB in a mouse experiment, an increase in antigen-specific suppressor T cells (Foxp3⁺CD25⁺CD4⁺ Treg) is reported [10].

To evaluate the allergic inflammatory response, we determined the infiltration of eosinophils and CD4-positive lymphocytes. The density of both types of cells showed a significant increase in the allergy group compared with the other two groups. The CTB group showed no significant difference in cell density of both types of cells compared with the control group, indicating that antigens used with CTB can suppress allergic inflammation, including eosinophilic migration. ECP is an eosinophilic granule-derived protein in eosinophils. Determining ECP in tears may be a useful index to evaluate immunotherapy efficacy [8, 11].

Regarding the cytokine and chemokine profile, a significant increase was observed in the thymus-expressed chemokine (TECK/CCL25), which is involved in the “homing phenomenon” of antigen-sensitized lymphocytes in mucosa-associated lymphoid tissue (MALT) of the intestine [12–14] and in B-lymphocyte chemoattractant (BLC/CXCL13), which is reported to be synthesized by helperTh17 [15]. Therefore, it can be stated that immunotherapy using antigens with CTB activates B-cell-associated immune response with secretory IgA (sIgA), as the ocular surface plays a role in lymphoid tissue involved in mucosal immunity, called CALT [16–18], and is one part of MALT. This immune system is B-cell dominant, and

sIgA is a major component. Antigens are trapped by the lymphoepithelium in the conjunctiva and recognized by B cells in the lymphoid follicle in the CALT. Then, sensitized B cells enter the blood circulation through the thoracic duct, and homing on the ocular surface, they produce antigen-specific sIgA. This circulation route is called the common mucosal immune system (CMIS). An increase in TECK indicates activation of the homing phenomenon in CMIS and an increase of antigen-specific sIgA production. This is the inhibitory reaction of the antigen. It is reported that antigen immunotherapy using CTB plays a role in inducing antigen-specific sIgA [19], suppressing specific IgE antibody [9], and inducing regulatory T cells [10]. In this study, it was thought that histological changes, such as CALT expansion, IgA-positive cell increase in CALT, and increase of chemokines, such as TECK in the CTB group, were similar to previous reports about antigen-specific sIgA. Furthermore, according to the study on adjuvant effects of CTB, it is reported that CTB promotes IL-4 production from CD4-positive cells [20]. However, further examination is necessary because the increase of IL-4 does not act in concert with alteration in CD4-positive cells in the CTB group. In addition, we did not determine antigen-specific sIgA in this study. As was done in the study of patients with allergic conjunctivitis [21], further investigations will be required to determine sIgA in tears of mice after allergen-specific immunotherapy.

Our study revealed that expression of GATA-3 mRNA, a specific molecular marker of Th2 cells, was positive in conjunctival tissue. Therefore, it is possible that infiltrated CD4-positive T cells are the same as Th2 cells. However, expression of both T-bet mRNA—which relates Th1 cells and Foxp-3, and mRNA to regulatory T cells, was negative. It was not clear whether regulatory T cells were involved in this study of local immunotherapy in experimental allergic conjunctivitis.

This study revealed that transconjunctival immunotherapy with CTB as an adjuvant has potential effects on allergic inflammation suppression in the conjunctiva. Thus, transconjunctival immunotherapy using CTB can be evaluated by histological examination of eosinophils and CD4-positive T cells, with mucosal immunity-associated chemokine and Th17-associated chemokine as biomarkers.

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References

1. Rolland JM, Gardner LM, O’Hehir RE, et al. Allergen-related approaches to immunotherapy. *Pharmacol Ther.* 2009;121: 273–84.

2. Saito K, Shoji J, Inada N, Iwasaki Y, Sawa M, et al. Immunosuppressive effect of cholera toxin B on allergic conjunctivitis model in guinea pig. *Jpn J Ophthalmol*. 2001;45:332–8.
3. Shoji J, Sakimoto T, Muromoto K, Inada N, Sawa M, Ra C, et al. Comparison of topical dexamethasone and topical FK506 treatment for the experimental allergic conjunctivitis model in Balb/c mice. *Jpn J Ophthalmol*. 2005;49:205–10.
4. Cohen SG, Frankland AW, Dworetzky M, Noon and Freeman on prophylactic inoculation against hay fever. *J Allergy Clin Immunol*. 2003;111:1572.
5. Li G, Liu Z, Zhong N, Liao B, Xiong Y, et al. Therapeutic effects of DNA vaccine on allergen-induced allergic airway inflammation in mouse model. *Cell Mol Immunol*. 2006;3:379–84.
6. Koizumi T, Abe T. Induction of oral tolerance to experimental allergic conjunctivitis in rats. *Nippon Ganka Gakkai Zasshi*. 1995;99:515–20.
7. Nunez JA, Cuesta U, et al. Local conjunctival immunotherapy: the effect of dermatophagoides pteronyssinus local conjunctival immunotherapy on conjunctival provocation test in patients with allergic conjunctivitis. *Allergol et Immunopathol*. 2000;28:301–6.
8. Ventura MT, Carretta A, Tummo RA, Buquicchio R, Arsieni A, Murgia N, et al. Clinical data and inflammation parameters in patients with cypress allergy treated with sublingual swallow therapy and subcutaneous immunotherapy. *Int J Immunopathol Pharmacol*. 2009;22:403–13.
9. Rask C, Holmgren J, Fredriksson M, Lindblad M, Nordström I, Sun JB, et al. Prolonged oral treatment with low doses of allergen conjugated to cholera toxin B subunit suppresses immunoglobulin E antibody responses in sensitized mice. *Clin Exp Allergy*. 2000;30:1024–32.
10. Sun JB, Cuburu N, Blomquist M, Li BL, Czerkinsky C, Holmgren J, et al. Sublingual tolerance induction with antigen conjugated to cholera toxin B subunit induces Foxp3⁺ CD25⁺ CD4⁺ regulatory T cells and suppresses delayed-type hypersensitivity reactions. *Scand J Immunol*. 2006;64:251–9.
11. Shoji J, Kitazawa M, Inada N, Sawa M, Ono T, Kawamura M, et al. Efficacy of tear eosinophil cationic protein level measurement using filter paper for diagnosing allergic conjunctival disorders. *Jpn J Ophthalmol*. 2003;47:64–8.
12. Wurbel MA, Philippe JM, Nguyen C, Victorero G, Freeman T, Wooding P, et al. The chemokine TECK is expressed by thymic and intestinal epithelial cells and attracts double- and single-positive thymocytes expressing the TECK receptor CCR9. *Eur J Immunol*. 2000;30:262–71.
13. Zabel BA, Agace WW, Campbell JJ, Heath HM, Parent D, Roberts AI, et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes, and is required for thymus-expressed chemokine-mediated chemotaxis. *J Exp Med*. 1999;190:1241–56.
14. Hosoe N, Miura S, Watanabe C, Tsuzuki Y, Hokari R, Oyama T, et al. Demonstration of functional role of TECK/CCL25 in T lymphocyte-endothelium interaction in inflamed and uninfamed intestinal mucosa. *Am J Physiol Gastrointest Liver Physiol*. 2004;286:G458–66.
15. Takagi R, Higashi T, Hashimoto K, Nakano K, Mizuno Y, Okazaki Y. B cell chemoattractant CXCL13 is preferentially expressed by human Th17 cell clones. *J Immunol*. 2008;18:186–9.
16. Knop N, Knop E, et al. Conjunctiva-associated lymphoid tissue in the human eye. *Invest Ophthalmol Vis Sci*. 2000;41:1270–9.
17. Inada N, Shoji J, Takaura N, Sawa M, et al. A morphological study of lymphocyte homing in conjunctiva-associated lymphoid tissue. *Nippon Ganka Gakkai Zasshi*. 1995;99:1111–8.
18. Sakimoto T, Shoji J, Inada N, Saito K, Iwasaki Y, Sawa M, et al. Histological study of conjunctiva-associated lymphoid tissue in mouse. *Jpn J Ophthalmol*. 2002;46:364–9.
19. Kim HJ, Kim JK, Seo SB, Lee HJ, Kim HJ, et al. Intranasal vaccination with peptides and cholera toxin subunit B as adjuvant to enhance mucosal and systemic immunity to respiratory syncytial virus. *Arch Pharm Res*. 2007;30:366–71.
20. Wang M, Bregenholt S, Petersen JS, et al. The cholera toxin B subunit directly costimulates antigen-primed CD4⁺ T cells ex vivo. *Scand J Immunol*. 2003;58:342–9.
21. Inada N, Shoji J, Hoshino M, Sawa M, et al. Evaluation of total and allergen-specific secretory IgA in tears in allergic conjunctival disease. *Jpn J Ophthalmol*. 2007;51:338–42.