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Invited Review Article

Ocular allergy test and biomarkers on the ocular surface: Clinical test for evaluating the ocular surface condition in allergic conjunctival diseases

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ABSTRACT

Allergic conjunctival diseases (ACDs) are inflammatory diseases of the conjunctiva and cornea caused predominantly by the IgE-mediated immediate hypersensitivity response. Allergic conjunctival diseases include allergic conjunctivitis, vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and giant papillary conjunctivitis. In clinical practice of ACDs, an ocular allergy test using biomarker measurement is a crucial examination technique for diagnosing, evaluating severity, and determining the efficacy of medical treatment. The ocular allergy test includes the tear test for evaluating the concentration of biomarkers in tears and an ocular surface test for assessing the expression levels of messenger ribonucleic acid (mRNA) biomarkers on the ocular surface. The clinical usefulness of several biomarkers has been demonstrated in patients with ACDs; specifically, eosinophil cationic protein and eotaxin-2 as eosinophilic inflammation biomarkers; interleukin-4 and thymus and activation regulated chemokine (CCL17/TARC) as Th2 inflammation biomarkers; eotaxin, tumor necrosis factor- α and soluble IL-6 receptor as giant papillae biomarkers; and osteopontin and periostin as allergic inflammation and remodeling biomarkers. Furthermore, the ocular allergy test, quantitative evaluation methods using biomarkers have allowed for better understanding of the immunological and pathophysiological mechanisms of ACDs. Therefore, the search for a biomarker is important to make an ocular allergy test useful. In previous ocular allergy tests, the biomarkers for allergic inflammation in patients with chronic ACDs including VKC and AKC were substantial. However, the selection of biomarkers associated with the early phase reaction of immediate hypersensitivity and innate immunity responses needs to be addressed in future investigations.

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Introduction

Allergic conjunctival diseases (ACDs) are inflammatory diseases of the conjunctiva and cornea caused predominantly by the IgE-mediated immediate hypersensitivity response.¹ Allergic conjunctival diseases in Japan consist of four clinical types: allergic conjunctivitis (AC), atopic keratoconjunctivitis (AKC), vernal keratoconjunctivitis (VKC), and giant papillary conjunctivitis (GPC).² Each clinical form of ACD differs in the characteristics of clinical findings and in severity. Atopic keratoconjunctivitis is a severe form of keratoconjunctivitis with atopic dermatitis in the face and often develops atopic ocular complications such as keratoconus, atopic cataract, and retinal detachment.³ Vernal keratoconjunctivitis

affects children and young adults. The development of conjunctival proliferative alterations such as giant papillae and gelatinous limbal infiltration are characteristic clinical findings in patients with VKC.⁴ Three subtypes of VKC exist: (1) the tarsal form, which is characterized by giant papillae with cobblestone appearance at tarsal palpebral conjunctivitis; (2) the limbal form, in which severe limbal gelatinous infiltration with Trantas-Dots spots develop; and (3) the mixed form, in which giant papillae and limbal gelatinous infiltration develop. Based on the clinical findings of AKC and VKC, the patients with giant papillae or gelatinous limbal infiltration are commonly determined as severe cases. In contrast, AC is a form of conjunctivitis with non-atopic dermatitis and non-conjunctival proliferative alterations. It consists of a perennial form and a seasonal form. Seasonal allergic conjunctivitis (SAC) caused by Japanese cedar pollen antigen and perennial allergic conjunctivitis

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(PAC) caused by house dust mite antigen are representative clinical subtypes of AC in Japan.

The representative subjective symptoms of ACDs are ocular itching. In addition, the patients with ACDs also complain of congestion, eye discharge, foreign body sensation, and epiphora. The common objective findings of ACDs are conjunctival hyperemia, conjunctival edema and swelling. Severe types of ACDs with the conjunctival proliferative alterations are complicated with epitheliopathy or shield ulcer in the cornea and are recognized as ocular surface disorders. Therefore, the patients with ACDs require differential diagnosis for non-allergic ocular surface disorders including infectious conjunctivitis, dry eye, cicatricial conjunctivitis, and phlyctenular keratoconjunctivitis. The severity grading is one of the crucial clinical assessments for the indication of the medical treatment in patients with ACDs. However, the severity grading used in clinical settings to evaluate ACDs is insufficient for understanding the detailed allergic inflammatory exacerbation and recovery. Therefore, ocular allergy tests that enable a correct diagnosis, differential diagnosis, and evaluation of disease severity are indispensable test tools in ophthalmologic practice for ACDs.

The ocular surface that provides an anatomical barrier against the external environment is composed of conjunctival epithelium, corneal epithelium, and tear film covering the conjunctival and corneal epithelium.^{5,6} In addition, the ocular surface is provided with the non-specific defense mechanisms including lactoferrin, lysozyme, and anti-microbial peptides,^{5,7,8} and specific defense mechanisms for mucosal immunity consisting of a secretory immunoglobulin A (sIgA) and an sIgA producing lymphoid tissue named conjunctiva-associated lymphoid tissue.^{5,6,9} Therefore, in the ACDs affected by environmental antigen exposure, the ocular surface plays a key role in host–environment interface and is one of the important organs in ocular allergic inflammation. Although the ocular surface has no direct access to the blood and lymphatic circulation, the ocular allergy test is a crucial clinical examination for evaluating the allergological pathophysiology of the ocular surface. Similar organ-specific examinations such as airway epithelial lining fluid in patients with asthma¹⁰ and nasal lavage

fluid in allergic rhinitis¹¹ have been investigated. The combination of organ-specific and systemic allergic tests may lead to a good understanding of the allergic disease.

Ocular allergy test for ACDs

In previous development of the ocular allergy test, quantitative evaluation methods using biomarkers allow better understanding of the immunological and pathophysiological mechanisms of ACDs and evaluation of clinical severity and efficacy of medical treatment in patients with ACDs. Therefore, specimens and their sampling methods, and measuring methods for biomarkers are crucial in ocular allergy testing.

Tear collection and tear test

Tear specimens can be easily obtained from the ocular surface by using various sampling techniques. However, for a quantitative tear test, the establishment of a method to obtain an appropriate tear volume is important for evaluating the concentrations of ACDs-associated biomarkers in tears. In previous reports, microcapillary collection method,¹² filter paper method using Schirmer's test paper,¹³ surgical sponge methods,¹⁴ and aspiration techniques by glass capillary tube¹⁵ or pipettes¹⁶ have been used as tear sampling methods. However, these sampling methods included the following problems: tear attenuation by reflective tearing when sampling tears, alteration in the concentrations because of contamination by inflammatory cells and epithelial cells, tear elution techniques, and creation of tear samples. When the results of the tear test are compared, equalizing the tear sampling method is important.

We improved a simplified lacrimal sampling technique and the quantifiability of the volume of sampled tears by using the filter paper method with Schirmer's test paper (Fig. 1). One millimeter of wet Schirmer's test paper contains 1 μ L of tears. Moreover, approximately 80% of tears can be collected by using adequate elution solution (Fig. 2). Therefore, the Schirmer strips were eluted overnight at room temperature using 0.5 M NaCl and 0.5% Tween

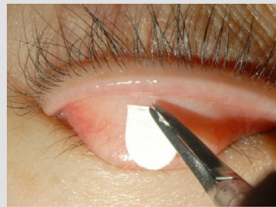
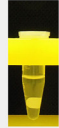
	Tear test	Ocular surface test
Sampling method	Schirmer's test 	Impression cytology 
Sample and Preservation method	-80 °C Tears containing inflammatory cells 	-4 °C The mucin and tears coating the ocular surface 
Measurement method	• Beaded assay • Antibody array • EIA, ELISA	• PCR array • Real-time PCR

Fig. 1. Ocular allergy tests using Schirmer's test paper. In the tear test, the tears are obtained from the lower conjunctival sac with a filter paper. The specimens from a patient with upper palpebral conjunctivitis are obtained by impression cytology using filter paper. EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

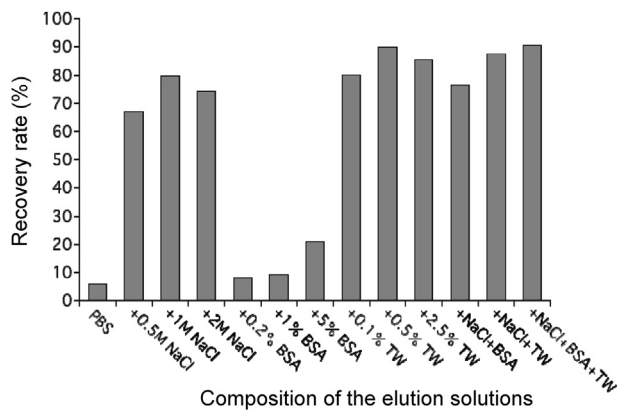


Fig. 2. Evaluation of the elution solution for tear test. The tear recovery rate from a filter paper varies depending on a composition of elution solution. PBS, 0.01 M phosphate-buffered saline; +0.5 M NaCl, 0.01 M phosphate-buffered saline plus 0.5 M sodium chloride; +1 M NaCl, 0.01 M phosphate-buffered saline plus 1 M sodium chloride; +2 M NaCl, 0.01 M phosphate-buffered saline plus 2 M sodium chloride; +0.2% BSA, 0.01 M phosphate-buffered saline plus 0.2% bovine serum albumin; +1% BSA, 0.01 M phosphate-buffered saline plus 1% bovine serum albumin; +5% BSA, 0.01 M phosphate-buffered saline plus 5% bovine serum albumin; +0.1% TW, 0.01 M phosphate-buffered saline plus 0.1% Tween 20; +0.5% TW, 0.01 M phosphate-buffered saline plus 0.5% Tween 20; +2.5% TW, 0.01 M phosphate-buffered saline plus 2.5% Tween 20; +NaCl + BSA, 0.01 M phosphate-buffered saline plus 0.5 M sodium chloride and 0.5% bovine serum albumin; +NaCl + TW, 0.01 M phosphate-buffered saline plus 0.5 M NaCl and 0.5% Tween 20; +NaCl + BSA + TW, 0.01 M phosphate-buffered saline plus 0.5 M sodium chloride, 5% bovine serum albumin, and 0.5% Tween 20.

20 containing 0.05 M phosphate-buffered solution (pH 7.2).¹³ However, a drawback is that the tear specimen eluted from Schirmer's test paper is a diluted tear solution; therefore, a highly sensitive assay must be used to measure biomarkers in this filter paper method.

Impression cytology and ocular surface test

In impression cytology, cytological diagnosis in the ocular surface, goblet cell density, and keratinization of conjunctival epithelial cells are histologically evaluated.¹⁷ We investigated the usefulness of impression cytology with Schirmer's filter paper to assess conjunctival allergic inflammation-associated gene expression as the ocular surface test (Fig. 1). In our experience, impression cytology results in milder pain and discomfort at the test site when using Schirmer's test paper than when using nitrocellulose membrane. In addition, Schirmer's test paper is able to be performed as noninvasive tests every time, and is easy to use for several times of sampling specimens from ocular surface.^{18,19} Quantitative reverse transcription polymerase chain reaction (qRT-PCR) enabled the genetic measurement of the expression levels of the allergic inflammation-associated mRNA that developed in the specimens obtained by Schirmer's test paper. Simultaneous examination of the tear test and the ocular surface test provides substantial pathophysiological and immunological information about allergic inflammation on the ocular surface of patients with ACDs. With regard to the simultaneous measurement of mRNA expression levels of allergic inflammation-associated factors on the ocular surface, Inada *et al.*¹⁸ reported different mRNA expression levels of the histamine receptor subclass in patients with AKC and VKC. Aso *et al.*¹⁹ investigated mRNA expression levels of cytokines and chemokines simultaneously, and indicated that at least two types of inflammatory reactions—IL-1 α -associated and CXCL8-associated inflammation and CCL24-associated and IL-16-associated inflammation—may be involved in the exacerbation of chronic ACDs.

Evaluation of allergic inflammation on the ocular surface, based on the ocular allergy test

Biomarkers for eosinophilic inflammation

In allergic diseases such as bronchial asthma, allergic rhinitis, atopic dermatitis, and ACDs, eosinophilic infiltration is a major pathophysiological finding, depending on the allergic inflammation. Therefore, eosinophil-associated factors, including eosinophil granule proteins, and eosinophilic infiltration-inducing chemokines are promising biomarker for allergic diseases.

Eosinophil cationic protein

Eosinophil cationic protein (ECP) is a cationic protein in the matrix of specific granules of eosinophils, and is involved in host defense responses by marked toxicity for parasitic worms, bacteria, and single-stranded ribonucleic acid (RNA) viruses.²⁰ In mucosal tissue with allergic inflammation, eosinophil granule proteins, including ECP, cause epithelial damage and subepithelial tissue injury.^{21,22}

In patients with ACDs, tear ECP levels of ACDs can be used as a tear test for evaluating the severity of allergic inflammation on the ocular surface. Previous reports^{12,13,23} reveal that ECP levels in the tears of patients with ACDs are higher than those of control individuals. Moreover, the increased levels of the ECP in tears tend to be low in AC and high in VKC and AKC^{13,23} (Fig. 3). In patients with AKC or VKC, tear ECP levels are significantly correlated with disease severity, as evaluated by a clinical score²⁴ (Fig. 4). These results indicate that severe eosinophil inflammation occurs on the ocular surface in patients with VKC/AKC. The tear ECP levels are suitable as a biomarker of allergic inflammation in ocular surface. However, several diseases with a small increase in the tear ECP levels are included for patients with AC. For example, in patients with SAC caused by Japanese cedar pollen, the tear ECP levels increases only slightly, even during the pollen scattering period.²⁵ Therefore, the grade classification of tear ECP levels is necessary to better understand the severity of allergy inflammation on the ocular surface. The five grades classification of tear ECP levels are as follows²⁴: "grade 1" indicates the normal range or mild AC; "grade 2" indicates severe AC and the stable stage of VKC/AKC; "grade 3" includes mild or moderate VKA/AKC; "grade 4" includes severe VKA/AKC; and "grade 5" includes very severe AKC/VKC (Fig. 4). Based on the grade of the tear ECP level classification, an adequate medical drug may be selected.

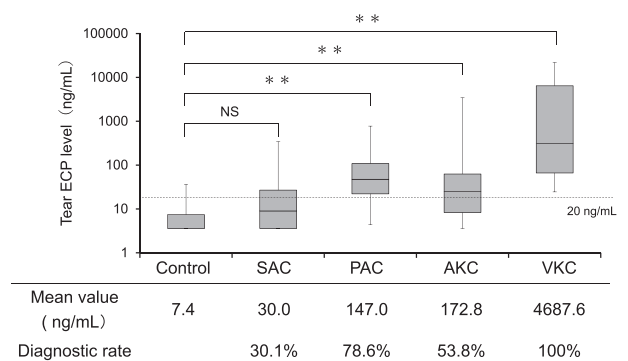


Fig. 3. Tear eosinophil cationic protein (ECP) levels in patients with allergic conjunctival diseases. The normal range of tear ECP levels is < 20 ng/mL. The positive rate was calculated by the proportion of positive specimens in which the tear ECP level was beyond 20 ng/mL. Steel test. ** $p < 0.01$. AKC, atopic keratoconjunctivitis; PAC, perennial allergic conjunctivitis; SAC, seasonal allergic conjunctivitis; VKC, vernal keratoconjunctivitis; NS, not significant.

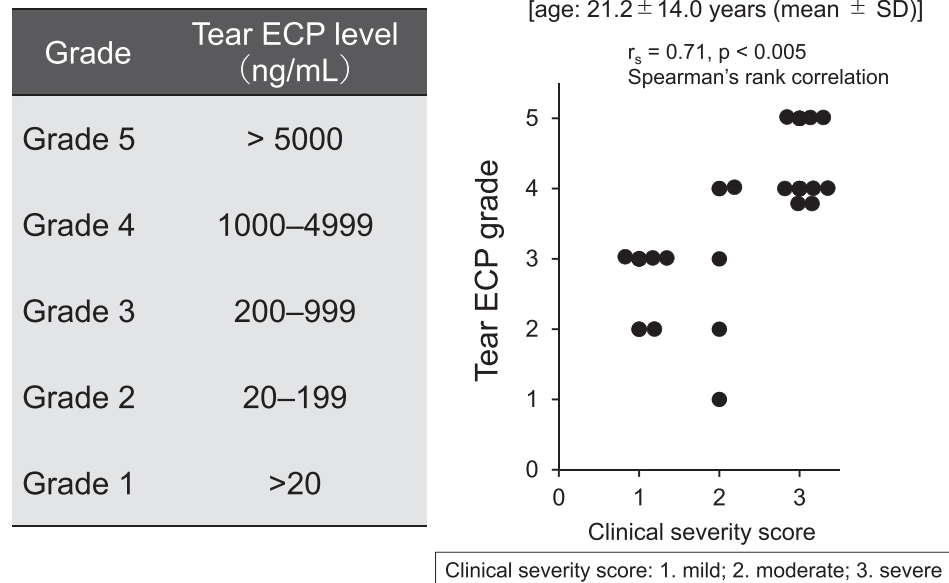


Fig. 4. Tear eosinophil cationic protein (ECP) grade. The tear ECP grade is significantly correlated with the clinical severity score in patients with vernal keratoconjunctivitis.

Eotaxin family

The eotaxin family, which includes eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26) is a C–C chemokine and is a ligand of C–C chemokine receptor type 3 (CCR3). C–C chemokine receptor type 3 is primarily expressed in eosinophils and basophils. In addition, CCR3 is also expressed in several different cell types, including Th2, mast cells, epithelial cells, and fibroblast²⁶; therefore, the eotaxin family may be major chemokines controlling inflammatory cells associated with allergic inflammation. Eotaxin-1 was first isolated from the lung lavage fluid of experimental asthma guinea pig model as an eosinophil chemotactic factor.²⁷ Thereafter, human eotaxin-1 was cloned, and its expression was confirmed in respiratory epithelium cells.²⁸ Eotaxin-1 expression is correlated with the early stage of eosinophil recruitment into the airway and skin after an allergen challenge.^{29,30} Eotaxin-2 is primarily produced from a monocyte/macrophage subset, and has chemotactic activity of eosinophils that is equivalent to that of eotaxin-1.³¹ Eotaxin-3 expression is limited to nonhematopoietic cells, and eotaxin-3 is expressed in vascular endothelial cells and in dermal fibroblasts after IL-4 and IL-13 stimulation. Moreover, eotaxin-3 mRNA expression is markedly increased in the airways of patients with asthma at 24 h after an allergen challenge.³² Therefore, eotaxin-2 and eotaxin-3 have similar function properties and are important for ongoing tissue eosinophilia.

The protein levels in tears and mRNA expression levels of eotaxin family on the ocular surface are reportedly increased in patients with ACDs than in normal individuals. Fukagawa *et al.*³³ report that eotaxin-1 in the tears of AKC patients with severe corneal damage is significantly increased, compared to the level in controls and AKC patients without corneal damage. An increase in eotaxin-1 in tears has also been reported in patients with SAC, and patients with contact lens-associated giant papillary conjunctivitis.^{34,35} High levels of eotaxin-1 and eotaxin-2 have been detected in the mucus of VKC patients, whereas only eotaxin-2 has been detected in the tears of patients with VKC and AKC.³⁶ In the analysis to detect eotaxin-1, eotaxin-2, and eotaxin-3 in the tears of patients with VKC, eotaxin-2 was the most detectable and highest concentrated eotaxin subfamily, whereas eotaxin-3 was not detected.³⁷ Eotaxin-2 levels in tears were significantly increased in the AKC

and VKC groups than in the AC and control groups (Fig. 5). In addition, eotaxin-2 levels in tears were significantly correlated with ECP levels in tears.^{37,38} (Fig. 5, Table 1). In double staining in immunohistochemical analysis, ECP and eotaxin-2 were both stained with eosinophils in conjunctival smear (Fig. 6). These results suggest that eotaxin-2 levels in tears are a suitable biomarker for eosinophilic inflammation on the ocular surface of ACDs patients as a substitute for the ECP levels in tears.

Furthermore, in the evaluation of eotaxin-1, eotaxin-2, and eotaxin-3 mRNA expression on the ocular surface, Inada *et al.*¹⁸ reported that among these three eotaxin subclasses, eotaxin-2 mRNA was the most commonly detected and showed the highest expression on the ocular surface in patients with AKC and VKC. Moreover, eotaxin-3 mRNA expression levels were significantly increased in patients with active stage of AKC and VKC, compared to the levels in patients with stable AKC and VKC (Fig. 7). Shiraki *et al.*³⁹ reported that the eotaxin-2 mRNA expression levels in patients with AKC or VKC were correlated with clinical scores that were assessed using a 5–5–5 exacerbation grading scale for allergic conjunctival diseases⁴⁰ (Table 2). With regard to the scoring of the clinical findings that were assessed using a 5–5–5 exacerbation grading scale for allergic conjunctival diseases, a clinical score represents the clinical severity of ACDs, and may become a biomarker in patients with ACDs. Therefore, eotaxin-2 levels in the tear test and eotaxin-2 mRNA expression levels on the ocular surface test are an excellent biomarker for evaluating eosinophilic inflammation in the conjunctiva of patients with AKC and VKC.

Histamine H₄ receptor

Histamine acts via the histamine receptors, which has been elucidated that four types of histamine receptor including H₁, H₂, H₃, and H₄ were expressed in conjunctival tissues.⁴¹ The H₁ receptor (H₁R) expressed in the conjunctival epithelial cells, goblet cells, C-fiber nerve terminals in conjunctiva, and vascular endothelial cells in subconjunctival tissues is a most common histamine receptor in IgE-mediated immediate hypersensitivity response. Ocular itching and conjunctival hyperemia are main clinical findings in patients with ACDs, and these clinical findings were related histamine activity via the H₁R.

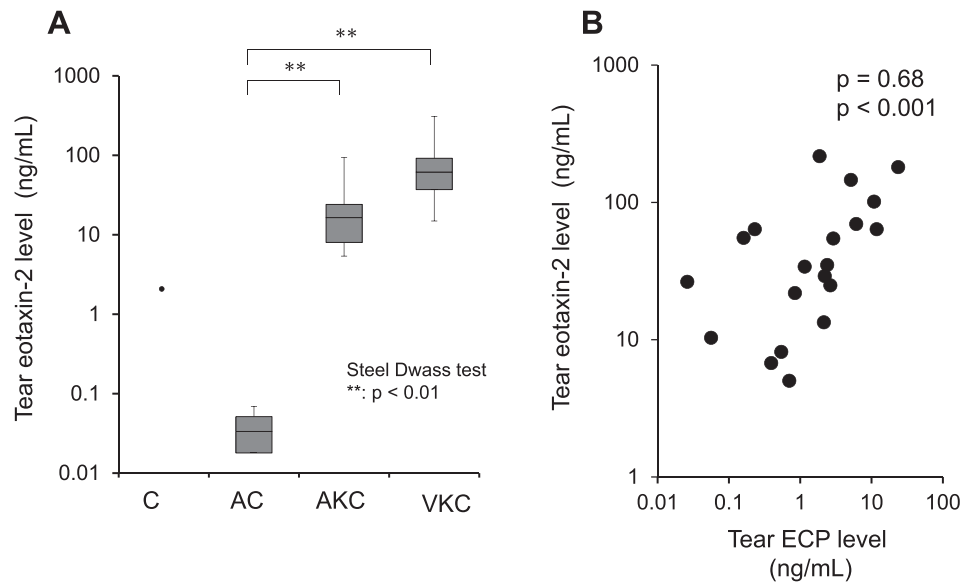


Fig. 5. Tear eotaxin-2 level in the patients with allergic conjunctival diseases. **(A)** Tear eotaxin-2 levels in the atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC) groups are significantly increased, compared to the levels in the allergic conjunctivitis (AC) group. **(B)** Tear eotaxin-2 levels are significantly correlated with tear ECP levels in patients with AKC or VKC ($n = 20$). C, control.

Histamine activity via the histamine H_4 receptor (H_4R) is predominantly expressed by immune cells and mediates inflammatory responses in the allergic reaction by selectively recruiting allergy-associated immune cells to the site of inflammation.^{42,43} In H_4R mRNA expression analysis using semi-quantitative reverse transcription polymerase chain reaction on the ocular surface, H_4R mRNA was expressed five times more in patients with VKC than in normal individuals.⁴¹ Furthermore, H_4R mRNA expression levels on the ocular surface were higher in patients during the active stage of AKC or VKC than in the control individuals.¹⁸ The expression levels of H_1R and H_4R mRNA were significantly correlated with the eotaxin-2 mRNA expression level¹⁸ (Table 1). In immunohistochemical staining for H_4R , Leonardi *et al.*⁴¹ reported that the H_4R was highly expressed in stromal inflammatory cells in conjunctival tissues. Inada *et al.*¹⁸ reported that eosinophils in conjunctival smear obtained from AKC or VKC patients were double stained with major basic protein and H_4R (Fig. 6). These reports indicated that the H_4R expressed on the ocular surface may be a convincing biomarker and therapeutic target molecule for allergic inflammation in ACDs.

Cytokine/chemokine profile in allergic inflammation of ocular surface

Th2 cytokine and Th2 chemokine

The helper T cell type 2 (Th2), which is a major lymphocyte in the conjunctival tissues with allergic inflammation, produces Th2 cytokines such as IL-4, IL-5, and IL-13 and commonly expresses C–C chemokine receptor (CCR)4 and CCR8. The major ligands for CCR4 are identified with CCL17/thymus and activation regulated chemokine (TARC) and CCL22/macrophage-derived chemokine (MDC). These ligands are Th2 chemokines. In particular, TARC levels in serum are a biomarker associated with the severity of the atopic dermatitis.⁴⁴

Th2 cytokine analysis in tear samples obtained from patients with ACDs, the concentrations of IL-4 are increased in patients with VKC and AKC with giant papillae.^{16,45} However, IL-4 in tears may be detectable only in patients with a severe case of AKC and VKC and is rarely detected in patients with SAC and PAC groups. On account

that a minimal volume of IL-4 is expressed in tears, measuring IL-4 is difficult. Therefore, the IL-4 levels in tears may not accurately reflect a pathologic condition of allergic inflammation in ocular surface.

In the examination for Th2 chemokines, TARC levels in tears of patients with AKC and VKC are significantly higher than the levels in patients with AC.³⁸ In patients with severe VKC, the elevation of tear TARC level was reportedly below the detection limit with medical treatment of the immunosuppressant eyedrops.³⁸ Therefore, TARC levels in tears may be useful as a marker of the acute exacerbation in patients with severe AKC or VKC.

Cytokine and chemokine profiles in tears

The simultaneous measurement of cytokines/chemokines in tears identified cytokine/chemokine profiles in the tears of patients with ACDs. The cytokines/chemokines profile in tears may reflect the pathophysiology of allergic inflammation on the ocular surface. Moreover, an ocular surface defense mechanisms including mucosal immunity are also involved in altering the cytokine/chemokine profile in tears.

Several reports exist regarding the pathophysiological analysis of the giant papillary formation, based on the cytokine profile. Leonardi *et al.*⁴⁶—based on cytokine/chemokine profiles in patients with SAC, VKC, and AKC by using multiplex bead analysis—reported that IL-4, interferon- γ , and IL-10 were elevated in SAC and VKC, while eotaxin and TNF- α were only increased in VKC. Furthermore, their analysis of culture supernatants of primary conjunctival fibroblasts indicated that IL-6, IL-8, monocyte chemoattractant protein-1, regulated on activation, normal T cell expressed and secreted (RANTES), and eotaxin were detected from fibroblast culture. The authors demonstrated striking similarities in chemokine profiles between tears and the culture supernatants, and suggested that the alteration of the cytokines in tears of the VKC patients are predominantly caused by conjunctiva fibroblasts.⁴⁶

We reported, based on cytokine profiles by antibody-array analysis, that the soluble IL-6 receptor was a significantly increased factor that was common in VKC and GPC (see Table 3).⁴⁷ Soluble IL-6 receptor (sIL-6R) is produced by proteolytic cleavage from the membrane-bound type of receptor (mIL-6R) or can be

Table 1

Eosinophilic inflammation-associated biomarkers in the tear test and the ocular surface test.

Biomarker	Correlation marker	Correlation coefficient	p-value	Reference
Eotaxin-2 (protein)	ECP (protein)	$\rho = 0.68$	$p < 0.005$	37, 38
Eotaxin-2 (mRNA)	Clinical score	$\rho = 0.80$	$p < 0.01$	39
H4R (mRNA)	Eotaxin-2 (mRNA)	$\rho = 0.83$	$p < 0.01$	18

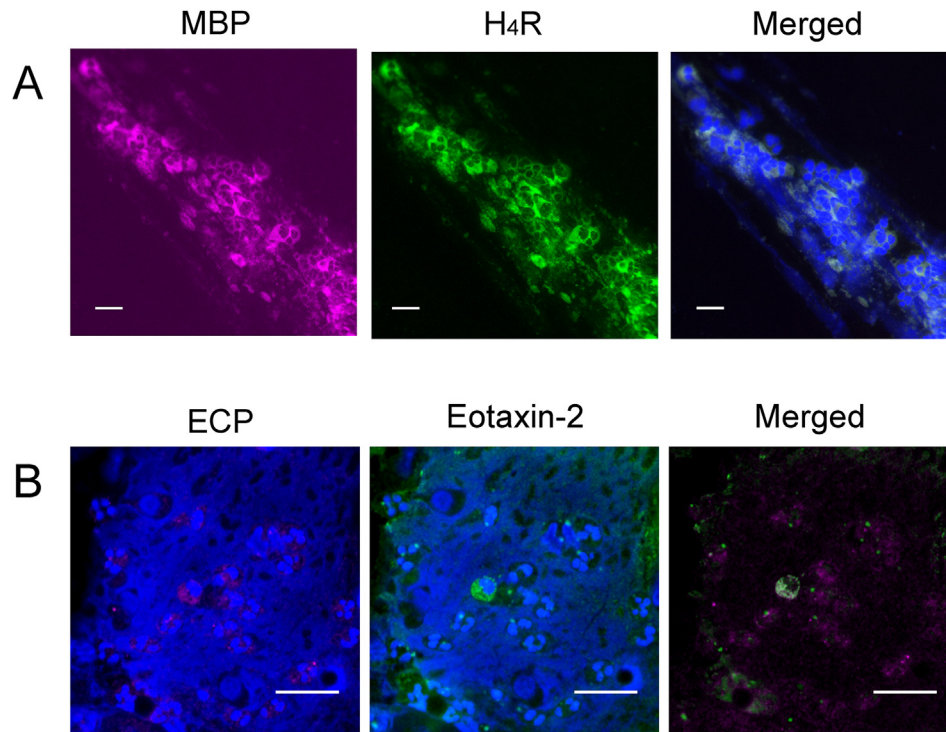
H4R, histamine H₄ receptor; ECP, eosinophil cationic protein.

Fig. 6. Immunohistochemical staining for eosinophils. Double-stained eosinophils with major basic protein (MBP) and histamine H₄ receptor (H4R) (A), and with eosinophil cationic protein (ECP) and eotaxin-2 (B) are present in the conjunctival smears of patients with vernal keratoconjunctivitis. The bar indicates 50 μ m. MBP, major basic protein; H4R, histamine H₄ receptor; ECP, eosinophil cationic protein.

generated by translation from alternatively spliced mRNA.⁴⁸ The sIL-6R is not an antagonist for IL-6 function. The IL-6/sIL-6R complex (i.e., trans-signaling responses) is capable of binding to gp130 and inducing intracellular signaling. Some research indicates that sIL-6R levels in the bronchoalveolar lavage fluid of patients with allergic asthma are increased at baseline, compared to these levels in the control study participants, and were further increased after an allergen challenge.⁴⁹ In a previous report,⁵⁰ the investigators suggested that IL-6 trans-signaling has a crucial role in the transition from acute to chronic phases of inflammatory processes. We evaluated sIL-6R levels in tears of patients with VKC, AKC without giant papillae, GPC, and control study participants. The sIL-6R levels were significantly higher in tears of patients with VKC and GPC than those in tears of individuals in the control group. Furthermore, sIL-6R levels in tears and the clinical score of allergic inflammation of the ocular surface were significantly correlated in patients with VKC.⁵¹ These results suggested that sIL-6R in tears is a suitable biomarker for conjunctival proliferative alterations such as giant papillary formation in chronic ACDs.

Pelikan⁵² reported that the allergic reaction occurring in the nasal mucosa can induce a secondary ocular response of an immediate, late, and delayed type in some patients with keratoconjunctivitis. The ocular response in patients with keratoconjunctivitis was analyzed by the simultaneous

measurement of chemical mediators, and cytokines in tears after a nasal allergen challenge. The concentration changes that were detected showed that histamine, tryptase, ECP, leukotriene (LT) C₄, prostaglandin (PG) D₂, PGF_{2 α} , IL-4, and IL-5 were increased in the immediate response; histamine, ECP, eosinophil-derived neurotoxin, LTB₄, LTC₄, PGE₂, myeloperoxidase (MPO), IL-4, and IL-5 were increased in the late response; and LTB₄, thromboxane B₂, MPO, interferon- γ , and IL-2 were increased in the delayed response. These concentration changes in chemical mediators and cytokines in tears indicate the similarity with those in patients with AC. Therefore, this investigation may be the report that clarifies the importance of integrated medical treatment for allergic rhinitis and ACDs.

Innate immunity-associated cytokines and chemokines

Type 2 inflammation in the allergic response consists of IgE-mediated immediate hypersensitivity and eosinophilic inflammation induced by IgE-independent group 2 innate lymphoid cell (ILC2). The ILC2 is recognized as the productive cells of Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13, and the highly expressed IL-33 receptor (ST2).⁵³ The epithelial cell-derived danger signal, including thymic stromal lymphopoietin (TSLP) and IL-33, activates the Th2 response and synergistically promotes ILC2 activation to induce type 2 inflammation. Matsuda *et al.*^{54,55} reported that TSLP

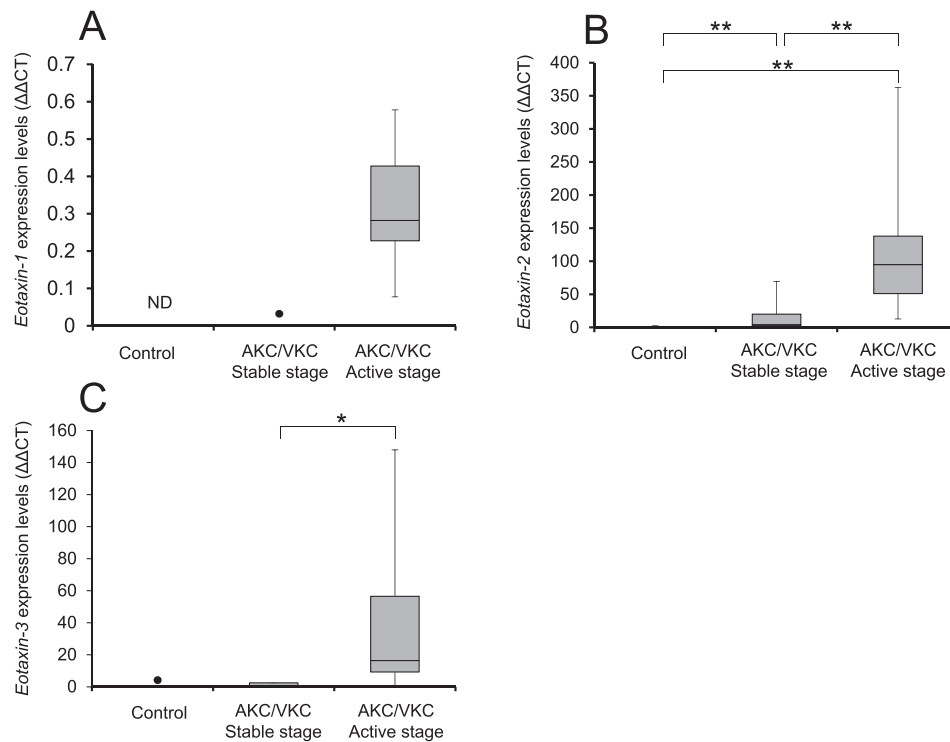


Fig. 7. Eotaxin-1, eotaxin-2, and eotaxin-3 messenger ribonucleic acid (mRNA) expression levels in patients in the active and stable stages of atopic keratoconjunctivitis/vernal keratoconjunctivitis (AKC/VKC). (A) Eotaxin-1 mRNA expression levels are not significantly different between the stable and active AKC/VKC patients and the control subjects. (B) Eotaxin-2 mRNA expression levels are significantly increased in patients in the active and stable stage of AKC/VKC, compared to the levels in the control participants, and eotaxin-2 mRNA expression levels in the active stage are higher than the levels in the stable stage. (C) Eotaxin-3 mRNA expression levels in stable AKC/VKC patients are only significantly increased. * $p < 0.05$; ** $p < 0.01$.

and IL-33 mRNA expression were detected with RT-PCR and the protein expression of TSLP and IL-33 were detected with immunohistochemical staining in giant papillae obtained from patients with AKC/VKC. With regard to the tear and ocular surface test for ILC2-associated type 2 inflammation in patients with ACDs, further investigation is required.

Other biomarkers for allergic conjunctival diseases

Osteopontin

In a previous report,⁵⁶ the combination of memory Th2 cells producing amphiregulin and osteopontin-producing eosinophils stimulated by amphiregulin via epidermal growth factor receptor facilitate airway fibrosis. Uchio *et al.*⁵⁷ report that the osteopontin levels in tears of AKC patients were significantly higher than the

levels in AC and control group individuals; the clinical severity of ACD was significantly correlated with the levels of osteopontin. These results suggested that osteopontin in tears is a promising marker of keratoconjunctival inflammation and the remodeling of the keratoconjunctival tissues in patients with severe and chronic ACDs.

Periostin

Periostin, an extracellular matrix belonging to the fasciclin family, has a critical role in the process of remodeling during tissue/organ development and repair.⁵⁸ In allergic diseases an increase in periostin expression in tissues is triggered by the IL-13 induced-allergic inflammation; and the overexpression of periostin in organs with allergic inflammation is involved in atopic dermatitis, allergic rhinitis, and eosinophilic esophagitis. Furthermore, in

Table 2

Judgment guidelines for the 5-5-5 exacerbation grading scale for allergic conjunctivitis disease.

The clinical sign grade	Exacerbation grading scale		
	100-point grade	10-point-grade	1-point-grade
Clinical sign	Active giant papillae [†] Gelatinous infiltrates of the limbus Exfoliative epithelial keratopathy Shield ulcer Papillary proliferation at the lower palpebral conjunctiva	Blepharitis Papillary proliferation with velvety appearance Horner-Trantas spots Edema of the bulbar conjunctiva Superficial punctate keratopathy	Papillae at upper palpebral conjunctiva Follicular lesion at lower palpebral conjunctiva Hyperemia of the palpebral conjunctiva Hyperemia of the bulbar conjunctiva Lacrimal effusion [‡]
Score	100 points × number of positive signs	10 points × number of positive signs	1 point × number of positive signs
Range	0–500 points	0–50 points	0–5 points

[†] "Active giant papillae" indicates that the giant papillary proliferation that spreads in a hemispherical shape with mucous discharge, but the mucous discharge is not essential. Giant papillae that are flat and have poor inflammation findings are excluded.

[‡] "Lacrimal effusion" refers to epiphora or lacrimal meniscus increase caused by an eye irritation symptom.

Table 3

Antibody array analysis for vernal keratoconjunctivitis and giant papillary conjunctivitis.

	VKC (vs Control)		GPC (vs Control)	
	Fold increase	p-value	Fold increase	p-value
Eotaxin	+	NS	ND	NS
Eotaxin-2	++	p < 0.05	++	NS
IL-4	++	NS	ND	NS
IL-6	++	NS	+	NS
sIL-6R	++	p < 0.01	++	p < 0.01
TIMP-2	++	NS	++	p < 0.05

Antibody-array was tested by Ray Bio™ Human Inflammatory Antibody Array III kit (RayBiotech, Inc., Norcross, GA, USA).

ND, no difference (less than four-fold increase or decrease, compared to the controls); NS, not significant; VKC, vernal keratoconjunctivitis; GPC, giant papillary conjunctivitis.

+, greater than four-fold increase; ++, greater than eight-fold increase, compared to the controls.

pediatric asthma, serum periostin levels are significantly higher in children with asthma than in control study participants, and are equivalent to conventional biomarkers.⁵⁹ Moreover, serum periostin levels are a biomarker for anti-IgE antibody therapy and anti-IL-13 antibody therapy.⁶⁰

In patients with ACDs, Fujishima *et al.*⁶¹ reported that the periostin level in tears is a good diagnostic tool for ACDs and is a suitable biomarker for evaluating the efficacy of medical treatment for VKC and AKC. Therefore, periostin is a promising biomarker in the tear test and examinations on its use as a biomarker of tear test are ongoing.

Conclusion

The ocular allergy test can be used to correctly evaluate the pathophysiology of the allergic reaction occurring on the ocular surface by using an adequate biomarker. However, the ocular allergy test has the following limitations: the number of the measurable biomarkers is limited because of the small volume of sampled specimens obtained, and the measurements of minor biomarkers in the sampled specimens become undetectable. Therefore, it is important to discover a biomarker that will make ocular allergy testing more useful and practical. In previous ocular allergy testing, the biomarkers for allergic inflammation in patients with chronic ACDs, including VKC and AKC, are substantial, but selection of the biomarkers for the early phase reaction of immediate hypersensitivity and the innate immunity are future issues for investigation.

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Conflict of interest

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