

Efficacy of Tear Eosinophil Cationic Protein Level Measurement Using Filter Paper for Diagnosing Allergic Conjunctival Disorders

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Purpose: We investigated the efficacy of the tear sampling method using a filter paper to evaluate eosinophil cationic protein (ECP) levels in patients with allergic disorders.

Methods: Subjects were an allergic group comprising patients with allergic conjunctivitis, vernal keratoconjunctivitis, or atopic keratoconjunctivitis, a Sjögren group comprising patients with secondary Sjögren syndrome, and a control group comprising healthy volunteers. Tears were sampled using the Schirmer Method I and the sample was eluted from the filter paper in 50 μ L of elution solution containing phosphate buffer solution with 0.5 M NaCl + 0.1% Tween 20. Then the ECP concentration in the elution sample was determined by the enzyme-linked immunosorbent assay method.

Results: Tear ECP level in the allergic group was significantly higher than the levels in the other two groups ($P < .01$ for the Sjögren group and $P < .001$ for the control). Furthermore, the tear ECP level of each allergic disease subgroup in the allergic group was significantly higher than that in the control group.

Conclusions: This method of determining tear ECP concentration is useful not only to diagnose allergic conjunctival disorders but also to evaluate their clinical stages. **Jpn J Ophthalmol** 2003;47:64–68 © 2003 Japanese Ophthalmological Society

Key Words: Allergic conjunctivitis, atopic keratoconjunctivitis, eosinophil, eosinophil cationic protein, vernal keratoconjunctivitis.

Introduction

Allergic conjunctival disorders are caused by an immediate type of allergic reaction and comprise allergic conjunctivitis, vernal keratoconjunctivitis, and atopic keratoconjunctivitis. Allergic conjunctival disorders are diagnosed by serum antigen specific IgE, prick, or scratch skin test. Results of an ophthalmological examination, such as specific keratoconjunctival findings by slit-lamp microscopy or detection of eosinophil in conjunctival smear, play an important role in definitive diagnosis.¹

Eosinophil contains specific granules in its cytoplasm. The structure of the granule is divided into two portions, core and matrix. The core portion contains major basic protein and the matrix contains eosinophil cationic protein (ECP), eosinophil-derived neurotoxin and eosinophil peroxidase. The total amount of ECP in 10^6 eosinophil is about 10 μ g.² ECP is contained only in eosinophil and is released in the degranulation process of eosinophil. Sputum ECP concentration in patients with asthma was reported to be high and to show a good correlation with their clinical stages. Therefore, tear ECP concentration may be an index for the infiltration and degranulation of eosinophils in conjunctiva.

Several methods to determine tear ECP concentration have been reported,^{3–5} but they have involved basic problems in securing sufficient tear volume for determination and in the methods of tear

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sampling. In this study, we investigated the overall efficacy of the tear sampling method using filter paper and the optimal conditions to determine ECP concentration in order to diagnose allergic keratoconjunctivitis.

Materials and Methods

Subjects

This study was approved by the Ethics Committee for Clinical Study of the Nihon University School of Medicine, and informed consent was obtained from all participants. The subjects were divided into three groups; an allergic group that comprised 24 patients with allergic keratoconjunctival disorders such as allergic conjunctivitis, vernal or atopic keratoconjunctivitis; a Sjögren group as a nonallergic disease group comprising 12 patients with secondary Sjögren syndrome; and a control group comprising 19 healthy volunteers. Some patients in the allergic group were examined several times and the total number of examined eyes was 60 (Table 1). In these 60 examined eyes, 19 showed superficial punctate keratopathy or shield ulcer at the time of tear sampling and were designated as the epitheliopathy (+) subgroup. The remaining 41 eyes had no epitheliopathy at the time of tear sampling and were categorized as the epitheliopathy (−) subgroup. Furthermore, the 60 examined eyes were also categorized depending on the topical treatment for allergy at the time of each tear sampling. The treated subgroup comprised 44 eyes and the nontreatment subgroup, 16 eyes (Table 2). In the Sjögren and the control groups, both eyes were examined; the difference between the right and left eyes was investigated in the control group.

For the statistical analysis, the Mann-Whitney *U*-test or the Fisher direct analysis method was applied, and a *P* value less than .05 was determined as significant.

Table 1. Demographics of Study and Control Subjects

	No. of Subjects	No. of Eyes	Mean Age ± SD	Ratio (M:F)
Allergic group	24	60	30.1 ± 16.3	14:16
AC	7	14	35.9 ± 21.4	4:3
AKC	11	26	34.6 ± 11.7	2:9
VKC	6	20	17.8 ± 8.4	5:1
Control group	19	19	31.9 ± 7.7	10:9
Sjögren group	12	24	62.8 ± 9.0	0:12

AC: Allergic conjunctivitis, AKC: atopic keratoconjunctivitis, VKC: Vernal keratoconjunctivitis.

Table 2. Treatment Modalities Employed in Allergic Group

Treatment	No. of Eyes
Topical mast cell stabilizer only	26
Topical steroid only	4
Topical mast cell stabilizer and steroid	8
Topical mast cell stabilizer and subconjunctival injection of steroid	6
Nontreatment	16
Total	60

Method of Tear Sampling

Tears were sampled for 5 minutes according to the Schirmer Method I using filter paper (Schirmer Tear Production Measuring Strips, Showa Pharmaceutical, Tokyo). The filter paper with tear sample was stored in a microtube (Assist Tube; Assist Trading, Tokyo) at −20°C until the determination of ECP concentration.

Determination of Tear ECP Concentration

After the stored filter paper was thawed at room temperature, 5-mm lengths were cut from the end of the paper, which had been inserted in the fornix. About 5 µL of tear sample had been absorbed in this cut-off portion of the filter paper. Then the tear sample was eluted using a 50-µL elution solution consisting of 0.01 M phosphate buffer solution (PBS) with 0.5 M NaCl and 0.5% Tween 20, at room temperature for 3 hours. Based on our preliminary study regarding optimal elution conditions, the recovery rates in PBS with 0.5 M NaCl and 0.5% Tween 20 exceeded 80%.

ECP concentration in the tear samples was determined by the enzyme-linked immunosorbent assay (ELISA) method using the AlaSTAT microplate ECP kit (Diagnostic Products Corporation, Los Angeles, CA, USA).

Results

Tear ECP Concentration

The upper detection assay limit of the present method was 180 ng/mL and the lower detection assay limit was 3.6 ng/mL. In the control group, there was no significant difference in tear ECP concentration between the right and left eyes (right eyes : left eyes ratio, 1.60 ± 1.53). Figure 1 shows ECP concentrations in the allergic, Sjögren, and control groups; the allergic group showed significantly higher values than the Sjögren (*P* < .01) and the control (*P* < .001) groups

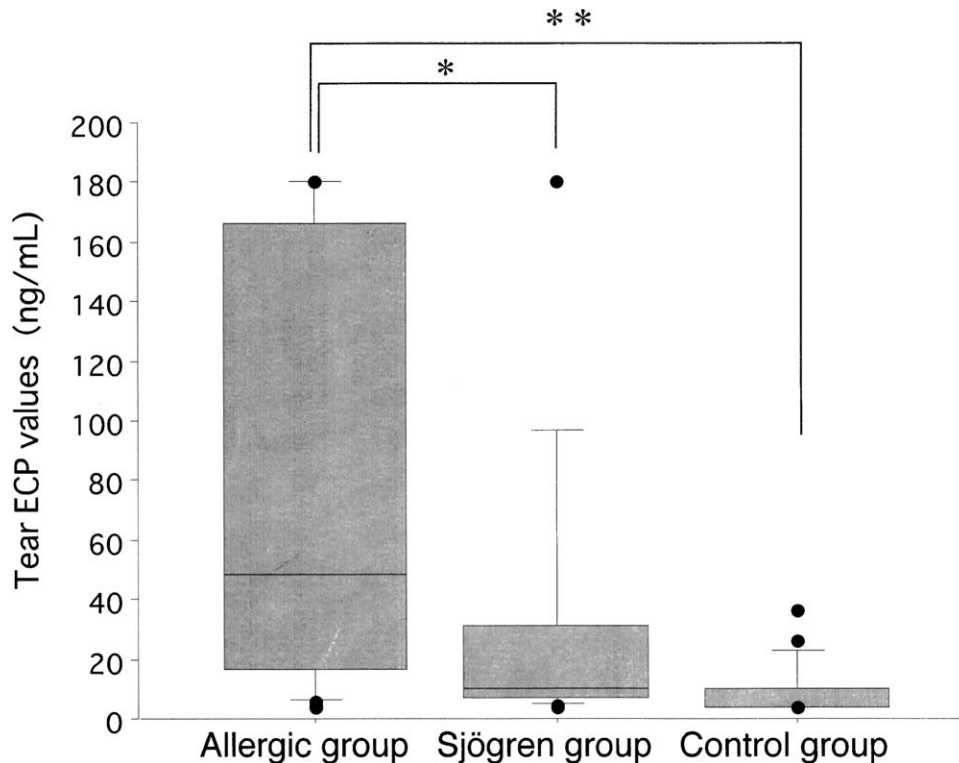


Figure 1. Tear eosinophil cationic protein (ECP) concentration. Tear ECP concentration in allergic group is significantly higher than that in Sjögren and control groups. * $P < .001$; ** $P < .01$, Mann-Whitney U -test.

(Mann-Whitney U -test). The allergic group was divided further into allergic conjunctivitis, vernal keratoconjunctivitis, and atopic keratoconjunctivitis subgroups. Some subjects exceeded the upper detection assay limit for ECP concentration; there were 2 in allergic conjunctivitis, 11 in vernal conjunctivitis, and 2 in the atopic keratoconjunctivitis group. On the other hand, 3 patients in the atopic keratoconjunctivitis subgroup were below the lower detection assay limit of concentration. ECP values for each subgroup were significantly higher than those for the control group ($P < .001$) (Mann-Whitney U -test) (Figure 2).

The cut-off level was set at 95 percentile ECP values for the control group, ie, 27.0 ng/mL. Examined eyes in each group were divided into two groups, belong the cut-off level. There was a significant difference among the three groups (Table 3).

In the allergic group, the epitheliopathy (+) subgroup (median value, 180 ng/mL) showed significantly higher ECP values than the epitheliopathy (–) subgroup (median value, 31 ng/mL) (Figure 3). The treated subgroups (median value, 11.5 ng/mL) showed significantly higher ECP values than the nontreated subgroup (median value, 63 ng/mL) (Figure 4).

Discussion

We investigated the efficacy of the tear sampling method using a filter paper to evaluate ECP levels in patients with allergic disorders.

ECP is a specific granular protein in the matrix portion of a specific granule in eosinophil and is released into tissues by the degranulation of eosinophil. The immediate type of allergic reaction is divided into early and late phases, and the late phase reaction is characterized by tissue infiltration by eosinophil and lymphocytes. A pathophysiologic study of allergic conjunctival disorders showed the infiltration of eosinophil in their late phase reactions. This phenomenon has also been revealed in an animal model for experimental allergy^{6,7} and in a follow-up study, an allergy evoked test⁸ in which eosinophil was identified in the tear or conjunctival smear. Furthermore, Montan et al⁹ reported the elevation of tear ECP concentration at 6, 8, and 24 hours after an allergen challenge test and confirmed the role of ECP in the late phase reaction. Therefore, it is thought that tear ECP determination has clinical significance in the diagnosis of allergic disorders and in the follow-up study of the stages of the disease.

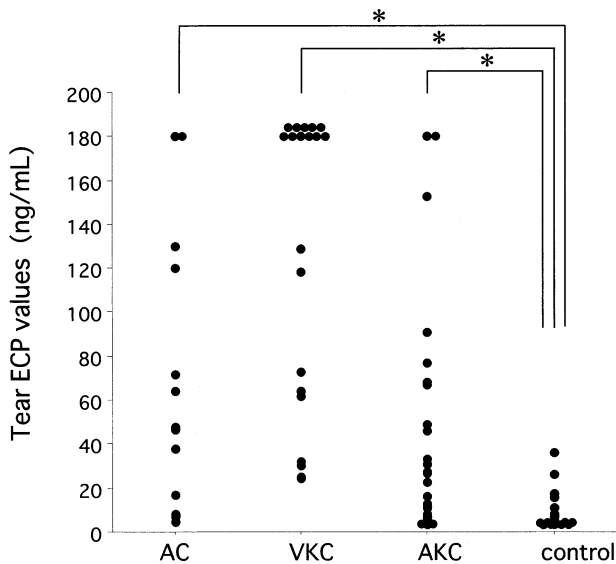


Figure 2. Comparison of tear eosinophil cationic protein (ECP) concentration among allergic disease subgroups and control group. Allergic disease subgroups (allergic conjunctivitis, vernal or atopic keratoconjunctivitis) show significantly higher ECP concentrations than the control group. * $P < .001$, Mann-Whitney U -test. AC: allergic conjunctivitis, VKC: vernal keratoconjunctivitis, AKC: atopic keratoconjunctivitis.

There have been several methods for sampling tear: the tear dilution method with irrigation solution, or filter paper, or microcapillary methods. Our results showed that the important factor for obtaining better results was not the filter paper method itself, but the ingredients of the elution solution used. The filter paper for the standard Schirmer test was convenient and safe for tear sampling compared to the other methods.

Our preliminary experiment regarding elution solutions revealed that the recovery rate of tear ECP from the filter paper depended on the composition of the elution solutions. Therefore, as the elution solution we chose PBS with 0.5 M NaCl and 0.1% Tween

Table 3. Fisher Direct Analysis of Tear Eosinophil Cationic Protein Values

	Allergic Group	Sjögren Group	Control Group
>Cut-off	40 eyes	7 eyes	1 eye
<Cut-off	20 eyes	17 eyes	18 eyes

Cut-off value: 27.0 ng/mL (95 percentile of control group)
Fisher direct analysis, * $P < .01$; † $P < .001$.

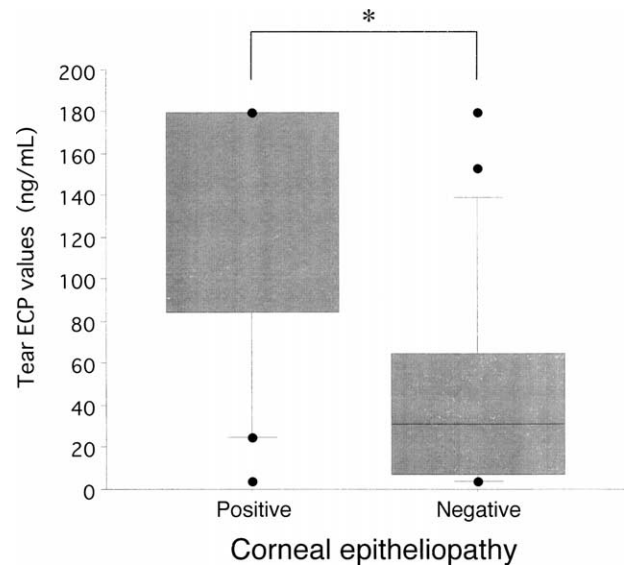


Figure 3. Tear eosinophil cationic protein (ECP) concentration in eyes with or without keratoepitheliopathy. ECP concentration in eyes with keratoepitheliopathy is significantly higher than in those without keratoepitheliopathy. * $P < .001$, Mann-Whitney U -test.

20, which gave a good recovery rate of more than 80%. We analyzed the ECP concentration in 25 μ L of elution solution using 5-mm lengths of the portion of filter papers which had been inserted in the con-

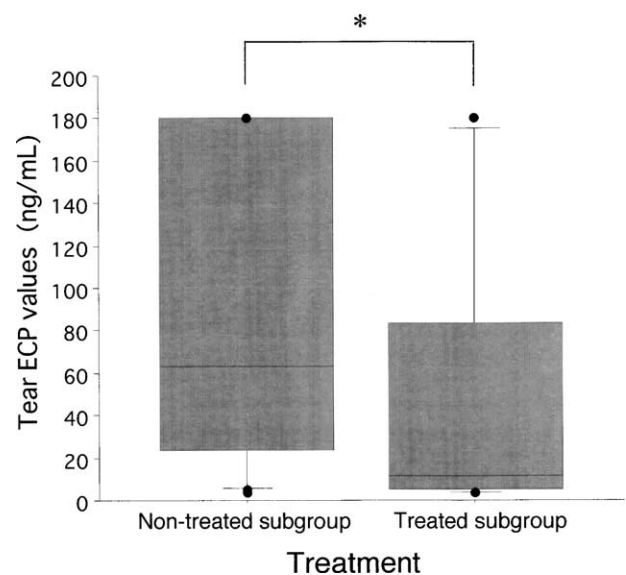


Figure 4. Tear eosinophil cationic protein (ECP) concentration depending on treatment history. Tear ECP concentration in eyes with topical mast cell stabilizer or immunosuppressive treatment is significantly lower than that in eyes without a treatment history. * $P < .05$, Mann-Whitney U -test.

junctival fornix. We expected that the inserted portion of the filter paper would yield the best tear sample. The dynamic range in concentration of the present method ranged from 3.6 to 180 ng/mL. In the ELISA method, if the sample exceeds its upper detection assay limit, it is analyzed after an appropriate dilution. However, since in this study our main objective was a comparison of tear ECP concentration among three groups, we did not dilute the elution solution further to avoid bringing the sample below the lower detection assay limit of the present method.

Concerning tear ECP value in the control group, Montan et al⁴ reported that most cases were less than 20 ng/mL. In addition, Leonardi et al³ reported that the mean tear ECP value for the control group was 7.5 ± 0.4 ng/mL. The tear ECP value for the control group in this study was less than 20 ng/mL except for 2 subjects. It is almost the same value as that in the previous reports. Tear ECP concentration in the allergic group was significantly higher than in the other two groups and this result agreed with the previous reports.^{3–10} They also reported that vernal and atopic keratoconjunctivitis showed remarkably high tear ECP values. Our results demonstrated that the allergic group showed significantly higher ECP levels than the non-allergic group, the Sjögren group.

The results of the comparative study among the allergic disease subgroups, with allergic conjunctivitis, vernal or atopic keratoconjunctivitis, and the controls, indicated that this method can be useful to make a definitive diagnosis of allergic disorders, regardless of the type of allergic disorder. The presumed cut-off level was set at the 95 percentile range of ECP concentration in the control group. Therefore, the determination of the cut-off level by data accumulated from a large number of additional control subjects is important to improve the accuracy of making differential diagnoses between allergic diseases and nonallergic disorders.

As can be easily understood from the pathophysiologic role of ECP in allergic reactions, its concentration decreases when degranulation of eosinophil does not occur. This situation might occur when sampling is not performed at the late phase reaction of immediate hypersensitivity, or at the high point of degranulation. On the contrary, it has been reported also that ECP concentration increased in patients with infectious conjunctivitis or rosacea keratitis.¹⁰

In this study, ECP concentration in the epitheliopathy (+) subgroup was significantly higher than that in the epitheliopathy (–) subgroup, indicating that ECP concentration did reflect the epithelial damage due to the late phase in the allergic reaction of the ocular surface. Furthermore, it was reported that ECP concentration decreased with the concurrent use of mast cell stabilizer or immunosuppressive drugs such as rodoxamine, dexamethasone, and cyclosporine.^{3,11} In this study, there was a difference in ECP concentration depending on the history of the drug therapies used (Figure 4). The result indicates that a follow-up study of ECP determination provide an index for evaluating the pharmacological effects of various drug regimens on allergic reaction.

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