

Elevated levels of substance P in tears of patients with allergic conjunctivitis and vernal keratoconjunctivitis

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Summary

Background Recent studies have suggested that the nervous system may participate in inflammatory processes. Substance P (SP) acts as a chemical mediator as well as a neurotransmitter.

Objective In order to clarify the pathogenesis of ocular allergic diseases, we assessed the concentration of SP in tears.

Methods Using a highly sensitive and specific double-antibody enzyme immunoassay (EIA), we determined the SP concentration in tears of 10 patients with seasonal allergic conjunctivitis, 10 with atopic dermatitis without keratoconjunctivitis (AD), 13 with vernal keratoconjunctivitis (VKC) and 65 normal controls. Giemsa's staining for brush cytology samples and histocytological study by immunocytochemical staining of giant papillary conjunctival cells from VKC and normal controls was conducted.

Results The mean SP level was low in the normal controls and AD, whereas patients with seasonal allergic conjunctivitis and VKC showed significant elevation of SP ($P < 0.01$). Brush cytology samples showed conjunctival epithelial cells with lymphocytes, neutrophils and eosinophils that were not seen in normal subjects. Histocytological examination demonstrated SP positive cells in the conjunctiva of patients with VKC, but not in normal controls.

Conclusion This study suggests that the increased level of SP in tears may contribute to the pathogenesis and severity of ocular allergic diseases.

Keywords: ocular allergic diseases, tears, substance P, enzyme immunoassay

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Introduction

The conjunctiva is a common site of allergic reactions due to its exposure to environmental allergens [1]. Nerves that are immunoreactive to substance P (SP) are found beneath and within the airway epithelium, and around the blood vessels and the submucosal glands [2]. Neuropeptides, such as SP, are widely distributed in the central and peripheral nerve endings, and are implicated as sensory

neurotransmitters and excitatory transmitters to intestinal muscles [3]. SP is involved in various physiological activities, and acts as a chemical mediator as well as a neurotransmitter [4,5]. The release of inflammatory cytokines such as IL-1, IL-6, and TNF- α from mononuclear cells by SP has also been reported [6]. Neuropeptides are believed to be involved in allergic reactions of the nasal mucosa via axon reflexes, neurogenic inflammation and interactions between the mast cells and nerves [7–10].

Since the contribution of SP in ocular allergic disease is unclear and we hypothesized SP production in ocular allergic disease would be different from normal, we measured

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the concentration of SP in tears from patients with seasonal allergic conjunctivitis, atopic dermatitis (AD) and vernal keratoconjunctivitis (VKC). We also studied SP-producing cells by means of an immunocytological study using giant papillary conjunctival tissue from patients with VKC.

Subjects and methods

Patients

The concentration of SP was determined in the tears of three different representative patient groups (seasonal allergic conjunctivitis, AD and VKC) of allergic disease and normal controls. Ninety-eight Japanese subjects (52 males and 46 females ranging in age from 10 to 58 yr, mean age $\pm$ SD 23.8 ± 7.5 yr) who visited the Department of Ophthalmology, Tokyo Dental College in Chiba, Japan, between February and March 1995, participated in this study. This is the relevant season for allergy to cedar pollen in Japan [11]. Diagnosis included seasonal allergic conjunctivitis (10 subjects, 31.6 ± 19.8 yr), AD (10 subjects, 25.8 ± 7.4 yr), and VKC (13 subjects, 16.7 ± 6.7 yr). Tears from 65 healthy individuals served as controls (44 males and 21 females aged 16 to 36 yr, mean 23.9 ± 2.6 yr). The diagnosis of allergic conjunctivitis was based on such symptoms and signs as: ocular itching, redness, tearing or pain; filamentous or mucous discharge, chemosis, hyperaemia or papillae of the palpebral conjunctiva on slit lamp examination; history; and positivity for serum antigen specific-IgE as indicated by the multiple antigen simultaneous test 16 (MAST16) [12,13].

The diagnosis of VKC was based on slit lamp examination findings of typical cobblestone excrescences (giant papillary conjunctivitis of the upper tarsal conjunctiva >1 mm in size) [14–16], and severe clinical symptoms such as keratitis and positivity for serum antigen specific-IgE. The diagnosis of AD was based on the diagnosis of dermatitis by a dermatologist, and by slit lamp examination showing the absence of keratoconjunctivitis, and no clinical symptoms indicative of allergic conjunctivitis. Control subjects had no symptoms or signs of allergic conjunctivitis, and tested negative for serum antigen-specific IgE antibodies. All subjects gave their informed consent for participation in this study.

Sample preparation and assay

A volume of tears exceeding 30 μ L was collected into Nunc minisorp tubes (Nunc, Roskilde, Denmark) using a micropipet without the use of solutions [17], and placed into chilled test-tubes containing 30 μ L aprotinin-EDTA mixture (500 kallikrein inhibitor units mL^{-1} aprotinin and 1.2 mg mL^{-1} EDTA). The tear samples were diluted 20-fold with

4% acetic acid, pH 4.0, and loaded onto reserved-phase C18 cartridges (Sep-Pak C18, Waters Co. Inc., Milford, MA, USA). After washing with 4% acetic acid, SP in tears was eluted with 70% acetonitrile in 0.5% acetic acid, pH 4.0. The elute was concentrated by spin vacuum evaporation, lyophilized, and stored at -40°C until assayed. Enzyme immunoassay for SP was performed as previously reported [3,18]. Antiserum RA-08-095 (Cambridge Research Biochemicals Ltd, Cambridge, UK) specific to the carboxyl-terminal portion of SP was used as the first antibody. EIAs were performed by the delayed addition method. Bound and free materials were separated by the double antibody solid phase method. The detectable minimum amount of SP was 0.4 fmol/well.

High performance liquid chromatography (HPLC) of tear extract

HPLC was performed using reversed-phase C18 column (Cosmosil 5C18-AR, 4.6×150 mm, Nacalai Tesque, Inc., Kyoto, Japan). The column was equilibrated with 0.1% trifluoroacetic acid. The tear sample (1.0 ml), purified by the Sep-Pak C18 cartridge as described above, were reconstituted to 200 ml with 0.1% trifluoroacetic acid and passed through the column. SP-like immunoreactive substances (SP-IS) were eluted with a linear gradient of acetonitrile (15% in 6 min and 15–50% in 35 min) in 0.1% trifluoroacetic acid. The flow rate was 1 ml/min and the fraction size was 1 ml. Synthetic SP and SP-sulphoxide (SP(O)) were applied to the column under the same conditions. Each fraction was concentrated by spin-vacuum evaporation and lyophilized. The residues were submitted to EIA.

Giemsa's staining for brush cytology samples

Conjunctival epithelial cells were collected by cytology brush from upper palpebral conjunctiva after topical anaesthesia [12,19]. Brush cytology samples were spread on slide glass, and fixation and Giemsa's staining were performed by May-Grunwald staining solution (Muto Pure Chemicals, Tokyo, Japan) and Giemsa's staining solution (Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan).

Biopsy of conjunctiva and immunohistochemistry

We made full-thickness horizontal incisions through the palpebral conjunctiva just posterior to the lid margin. After subconjunctival anaesthesia was administered, sharp dissection of the tarsal conjunctiva and the giant papillae from the tarsus was performed. The biopsied specimens were placed on a sterile aluminum sheet and cut with a razor blade and frozen with OCT compound until the histological examination.

The biotin-avidin immunoperoxidase staining method was performed on acetone-fixed cryostat sections of conjunctival biopsies. Briefly, they were stained with rat monoclonal antibody (MoAb) to human cell surface antigens for rat anti-SP (Chemion International Inc., Temecula, CA, USA), then with biotin rabbit anti-rat Ig (Zymed, Laboratories Inc., San Francisco, CA, USA), and finally, with streptavidin-horseradish peroxidase (Nichirei, Tokyo, Japan) and 3,3'-diaminobenzadine (Nichirei, Tokyo, Japan). Antibody was diluted to optimize staining results. Sections were routinely counterstained with haematoxylin.

Statistical methods

Data were reported as mean $\pm$ SD pg/mL. The level of SP in tears and age were analysed using Scheffe test, because it is not uniform in four groups by analysis of ANOVA ($P=0.0001$). A level of $P<0.05$ was accepted as statistically significant.

Results

Background of patients with VKC

Patients with VKC were younger (mid-teens) than those with seasonal allergic conjunctivitis (early 30s) ($P=0.0001$) and the normal controls ($P=0.02$). Patients with seasonal allergic conjunctivitis were older than the normal controls ($P=0.03$).

HPLC of tear extracts

For biochemical characterization of SP-IS, tear-extracts were applied to reversed-phase HPLC. The main SP-ISs in the tears from normal subjects were eluted at the same

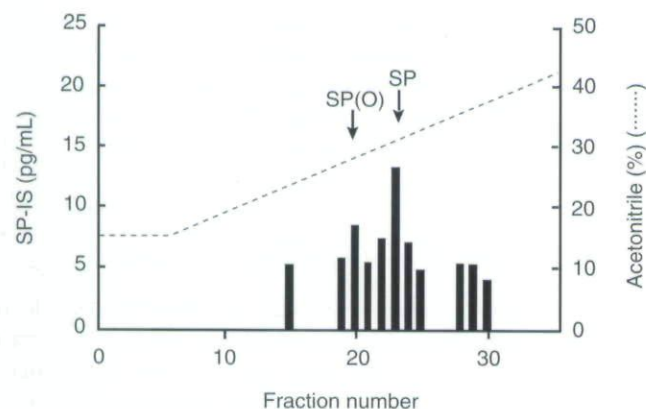


Fig. 1. HPLC elution profile of tear extract of normal subjects. The arrows indicate the elution regions of synthetic substance P (SP) and SP sulphoxide (SP(O)). Fraction number appears horizontally, and SP-IS (pg/mL) and acetonitrile (%) (---) appear vertically.

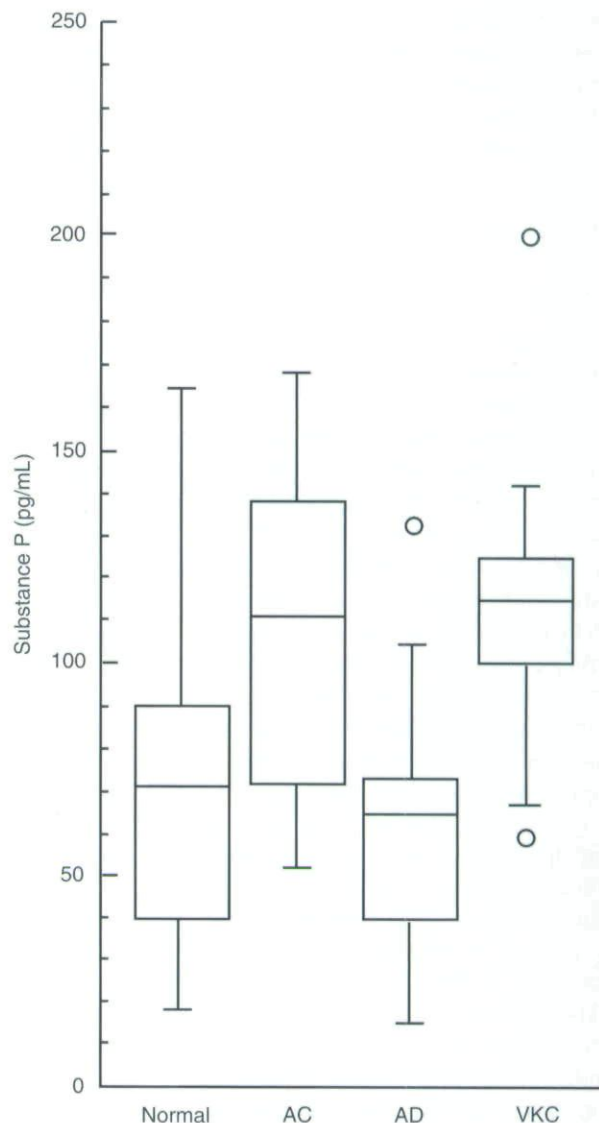


Fig. 2. Concentration of substance P in tears of patients with three different forms of conjunctivitis and in normal controls. Box plots show range of inter-quartile distance (IQD); distance between upper quartile (UQ) and lower quartile (LQ) with the median as the horizontal line; top shows median+ UQ+ (1.5X IQD) and bottom shows median-LQ- (1.5X IQD). Any responses outwith this range are shown as a circle (Scheffe test). AC=seasonal allergic conjunctivitis; VKC=vernal keratoconjunctivitis; AD=atopic dermatitis. SP in tears in patients with seasonal allergic conjunctivitis ($P=0.02$) and VKC ($P=0.01$) was significantly higher than in normal controls.

elution volume of the synthetic SP and SP(O) in the region of 33% and 29% acetonitrile respectively, with small minor peaks (Fig. 1). The assay of the tear-extracts was considered to be specific.

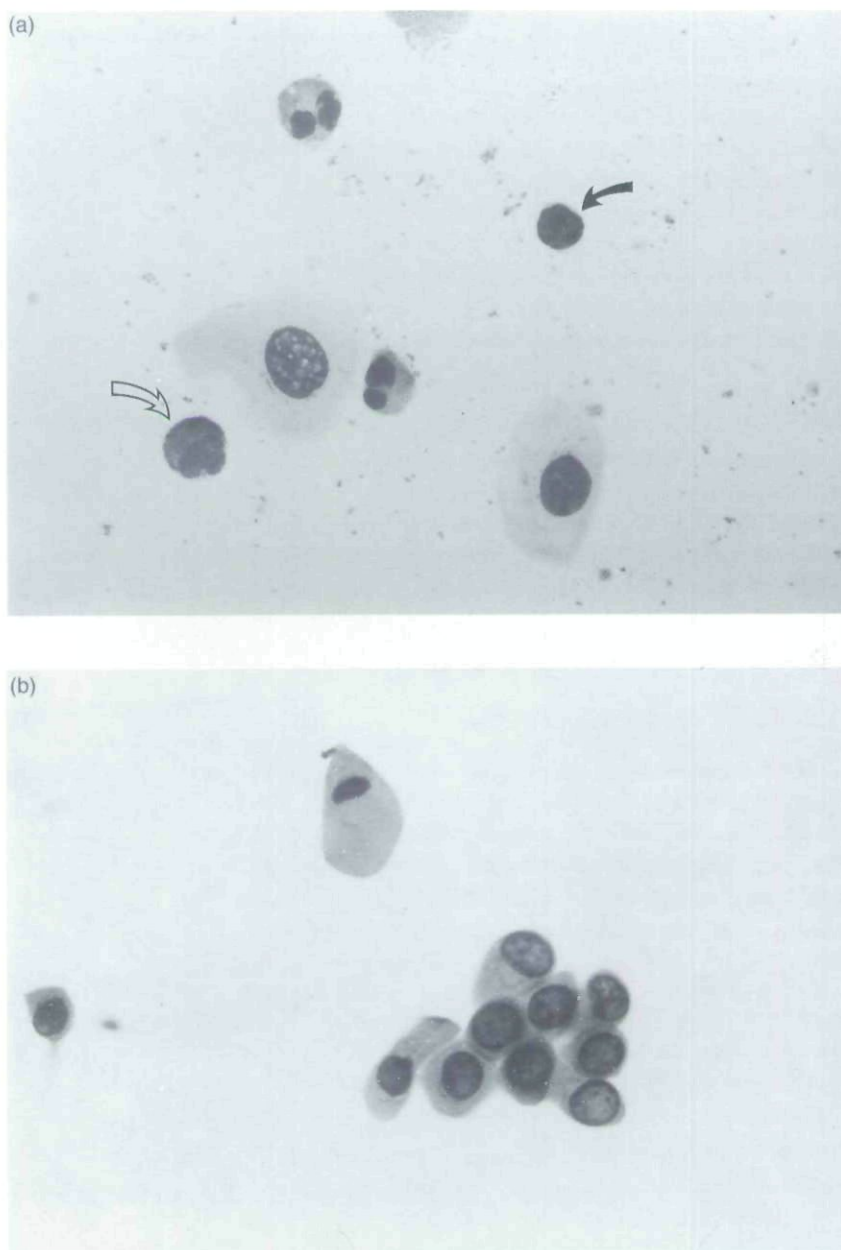


Fig. 3. Brush cytology samples of superior palpebral conjunctiva stained by May-Grunwald staining solution and Giemsa's staining solution. (a) Lymphocytes, neutrophils, eosinophils and conjunctival epithelial cells are observed. Black arrows indicate lymphocytes, white arrow indicates eosinophils. (b) Positivity was lacking in the specimens from normal subjects and 100% conjunctival epithelial cells.

SP in the tears

The mean level of SP in the tears of normal controls ($n=65$) was 70.9 ± 34.8 pg/mL (mean $\pm$ SD), in those with seasonal allergic conjunctivitis ($n=10$) it was 107.7 ± 37.3 pg/mL, in patients with AD ($n=10$) it was 65.0 ± 34.2 pg/mL, and in VKC patients ($n=10$) it was 114.4 ± 35.1 pg/mL (Fig. 2). Patients with seasonal allergic conjunctivitis ($P=0.03$) and VKC ($P=0.001$) had significantly higher mean levels of SP in tears than normal controls.

Histocytological study of conjunctival epithelial cells

May-Grunwald-Giemsa staining of brush cytology samples showed conjunctival epithelial cells with lymphocytes, neutrophils and eosinophils that were not seen in normal subjects (Fig. 3).

Immunohistological analysis of biopsy samples

Cryostat frozen sections were examined by immunohistochemical analysis. Giant papillary conjunctiva obtained

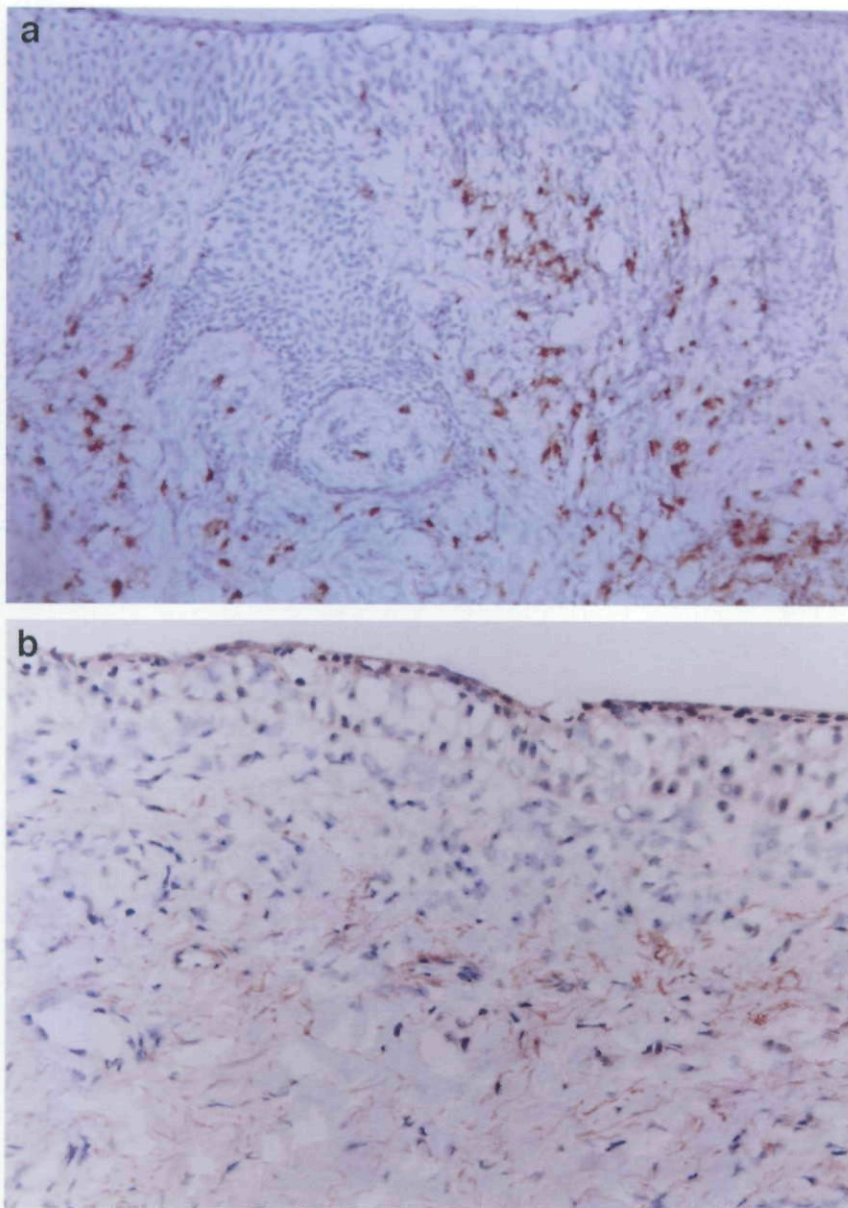


Fig. 4. Immunohistological analysis of conjunctival biopsies. Monoclonal antibodies were used to analyse frozen tissue sections of conjunctiva after acetone fixation. (a) Representative tissue from VKC patients' giant papillary conjunctiva. SP positive cells were seen in epithelial and subepithelial cells. (b) Representative tissue from conjunctiva of normal subject. No positive cells were observed in epithelium and subepithelium, but some local staining demonstrated around nerve fibres in subconjunctival tissue.

from VKC patients showed positivity for SP. Most of these stainings were localized in epithelium and subepithelium. Samples from normal subjects did not show the SP positive cells in epithelium and subepithelium, but some local staining was seen by the nerve fibres in subconjunctival tissue (Fig. 4).

Discussion

The increased level of SP in tears was clearly observed in all samples from severe allergic conjunctivitis. However, its precise contribution in allergic reactions is unclear. Allergic

reactions involving the nasal mucosa and conjunctiva are associated with histamine and other chemical mediators. Histamine depolarizes the sensory nerves containing histamine receptors, and the neural wave of depolarization is centrally converted to the secretomotor and itchy centres of the brain. From these centres, nerve impulses are transmitted to the autonomic nerves that innervate the walls of the blood vessels and submucosal glands and induce the secretion of various neurotransmitters from the nerve endings. These neurotransmitters produce vasodilation, an increased permeability of the blood vessels, and a hypersecretion of the submucosal glands, producing symptoms

of the allergic reaction [10,20]. The increased level of SP in tears contributes to the pathogenesis of ocular allergic inflammation.

The presence of inflammatory cells, including mast cells and eosinophils, has been demonstrated in conjunctival samples from patients with allergic conjunctivitis and VKC [13,15,19]. Though Figure 3 (a) does not show the mast cells, we chose a case which showed a large number of inflammatory cells. The presence of mast cells might depend on the clinical stage and some early progress cases show mast cells. Recent studies have revealed that mast cells [9] and intestinal eosinophils [21,22], when activated, may play a role in the production of SP. The eosinophil is thought to be importantly involved in the allergic reaction, and to be stimulated by various neuropeptides [8,9,21–23]. Our findings support those reported previously regarding the key role of SP in the development of allergic disorders, such as allergic conjunctivitis.

Immunohistochemical analysis revealed the presence of SP-positive cells in conjunctiva which were highly observed in VKC patients' giant papillary conjunctival samples, suggesting their involvement in the pathogenesis of allergic conjunctivitis and VKC. Previous studies demonstrated a marked increase of cytokine in inflammation and in the expression after the topical administration of substance P [7,19]. Therefore SP may regulate the inflammation caused by the allergic reaction. If so, pharmacological manipulation of SP levels may offer a new strategy for treating allergic diseases of the eye.

To our knowledge, there are no published studies on SP in tears, perhaps because of the difficulty in working with a small volume of tears and in detecting the low concentration of SP present in tears. A newly developed enzyme-linked immunoassay has proved useful in measuring small amounts of protein. While a volume of 30 μ L of tears was sufficient for quantifying SP, a lower volume can lead to unreliable results [17,24].

Tears are commonly collected in cellulose sponges, capillary tubes, or by aspiration. There are no statistically significant differences between these methods in terms of volume of collection, contamination and reflex lacrimation [24]. If the patients with conjunctivitis have elevated tear production due to irrigation, tear flow rates and volumes would support an elevated SP level, despite the dilution. As Tuft pointed out, any technique is subject to error, which may affect the obtained value and the dilution concentration of tears during their collection [24].

We found significantly increased SP concentrations in the tears collected from patients with seasonal allergic conjunctivitis and VKC as compared with control subjects and patients with AD. This increase may be disease related, not age related. While patients with AD were recruited as controls, since this decrease is caused by allergic reactions,

their SP levels were not increased. This might be because they had no ocular abnormalities.

We found increased levels of SP in the tears collected from patients with seasonal allergic conjunctivitis and VKC. While previous studies have shown that SP is produced by mast cells [9] or eosinophils [21,22], additional research is needed to identify the source of SP in patients with allergic disorders of the eye.

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