

Cellular changes in tears associated with keratoconjunctival responses induced by nasal allergy

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Abstract

Background Allergic keratoconjunctivitis occurs in a primary form, caused by an allergic reaction localized in the conjunctiva, and in a secondary form, induced by an allergic reaction originating in the nasal mucosa. Various hypersensitivity mechanisms involved in the keratoconjunctivitis forms result in different keratoconjunctival response types.

Purpose To investigate the cytologic changes in tears during the secondary immediate (SIKCR), late (SLKCR), and delayed (SDYKCR) keratoconjunctival responses.

Methods In 61 patients, comprising 20 SIKCRs, 23 SLKCRs, and 18 SDYKCRs, nasal provocation tests (NPTs) with allergens and 61 phosphate-buffered control challenges were repeated and supplemented with cell counting in the tears.

Results The SIKCR ($P < 0.01$), appearing 10–120 min after the NPT, was associated with increased eosinophil and mast cell counts in tears. The SLKCR ($P < 0.01$), appearing 5–12 h after the NPT, was accompanied by increased counts of eosinophils, neutrophils, basophils, and conjunctival epithelial and goblet cells. The SDYKCR ($P < 0.05$), appearing 24–48 h after NPT, was associated with increased counts of lymphocytes, neutrophils, monocytes, basophils, conjunctival epithelial, corneal epithelial and goblet cells.

Conclusions The SIKCR, SLKCR, and SDYKCR, induced by nasal allergy, were associated with different cellular profiles in the tears. The cells, except mast, epithelial and goblet cells, displaying no intracellular changes, migrated probably from the conjunctival capillaries, in response to the factors released during the primary allergic reaction in the nasal mucosa and

subsequently penetrating into the conjunctiva. These results demonstrate a causal role of nasal allergy and diagnostic value of NPT combined with recording of ocular features and cellular profiles in tears in some keratoconjunctivitis patients.

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Introduction

Allergic keratoconjunctivitis is classically divided into vernal (VKC) and atopic keratoconjunctivitis (AKC).^{1–6} However, with respect to localization of the initial allergic reaction two KC forms can be recognized. In the primary keratoconjunctivitis form, the allergic reaction due to the exposure of conjunctiva to an external allergen occurs in the conjunctiva, followed by additional involvement of cornea. The secondary keratoconjunctivitis form is induced by the allergic reaction taking place in the nasal mucosa due to the exposure of nasal mucosa to an inhalant allergen.^{7–10}

Various hypersensitivity mechanisms, such as immediate (IgE-mediated) type, late type (type III), or delayed type (cell-mediated), can underlie the primary as well as the secondary form of keratoconjunctivitis, which may then result in the development of three basic keratoconjunctival response types, an immediate, a late, or a delayed type.^{2–26}

There is a dearth of data demonstrating the role of a nasal allergy in the conjunctiva, cornea and in the possible induction of the secondary keratoconjunctival response (SKCR).^{7–10,16,23,26} Moreover, little data are available to illustrate

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the cytologic changes in tears during the particular types of SKCR.^{7–10}

The purpose of this study, which is a continuation of our earlier work,^{7–10,27} was (1) to investigate the cytologic profiles and changes in the counts of individual cell lineages in tears during the particular types of SKCR, (2) to evaluate the significance of cytologic changes in tears for the possible role of various immunologic mechanism(s) underlying the particular types of SKCR.

Materials and methods

Patients

All of the 61 patients suffering from keratoconjunctivitis, referred to our Department of Allergology & Immunology (Institute of Medical Sciences 'De Klokkenberg', Breda, The Netherlands) for extensive diagnostic analysis and developing secondary keratoconjunctival responses to the routine nasal provocation tests (NPTs) with allergen, volunteered to participate in this study. These patients, 28 males and 33 females, 15–37 years of age (Table 1), suffered from VKC ($n = 25$) or AKC ($n = 36$). The 33 AKC and 17 VKC patients also had atopic dermatitis on the face and/or periorbital area. In 12 of these patients

Table 1 Characteristics of the keratoconjunctivitis (KC) patients

	KC patients (n = 61)			Control subjects (n = 15)
	SIKCR (n = 20)	SLKCR (n = 23)	SDYKCR (n = 18)	
Age (years)	22 ± 4	23 ± 7	21 ± 6	25 ± 5
Sex (M/F)	10/10	11/12	7/11	6/9
Disease history (years)	3.4 ± 1.5	3.5 ± 1.7	4.1 ± 0.9	0
Blood leukocyte count ($\times 10^9/l$) ^a	8.3 ± 1.2	8.5 ± 1.0	7.7 ± 0.6	7.4 ± 1.1
Blood eosinophil count ($\times 10^6/l$) ^b	336 ± 19	305 ± 21	282 ± 16	264 ± 29
Increased total IgE in the serum ^c	1	0	0	0
Positive specific IgE in the serum ^d	10	2	0	5
<i>Skin prick tests—response^e</i>				
Immediate	12	4	0	1
<i>Positive skin response^e</i>				
Immediate type	15	8	1	6
Late type	5	15	7	9
Delayed type	0	0	10	0
<i>Nasal provocation tests</i>				
Positive	20	23	18	15
Negative	12	15	6	9

Abbreviations: SDYKCR, secondary delayed keratoconjunctival response; SIKCR, secondary immediate keratoconjunctival response; SLKCR, secondary late keratoconjunctival response.

^aNormal value 4.0–10.0 $\times 10^9/l$.

^bNormal value $< 300 \times 10^6/l$.

^cNormal value < 500 IU/ml.

^dNormal value < 0.70 U/ml (ImmunoCAP).

^ePositive skin response to the relevant allergen (allergen producing positive nasal and ocular or nasal response only).

positive specific IgE in the serum to *Dermatophagoides pteronyssinus* ($n = 5$) or grass pollen ($n = 7$) was recorded. In 19 patients the 34 conjunctival provocation tests with inhalant allergens performed previously by ophthalmologists were negative. None of them suffered from other ocular disorders, infections, or systemic disease. All patients had normal intraocular pressure. They had previously been treated with systemic or topical H1-receptor antagonists, ocular glucocorticosteroids, and decongestants, however, without any substantial complaint improvement.

Patients underwent a routine diagnostic procedure, serving also as inclusion–exclusion criteria, consisting of a disease history, basic laboratory tests, bacteriological screening of the tears, skin tests with inhalant allergens, X-ray of the paranasal sinuses, nasoscopy, ophthalmoscopy, measurement of intraocular pressure, slit-lamp evaluation, vital staining with fluorescein, single-tear cytology, and conjunctival scraping.²⁸

This procedure revealed a positive history for nasal allergy, edematous nasal mucosa, positive skin tests with inhalant allergens, hyperlacrimation and conjunctival hyperemia, incidental eosinophil and conjunctival epithelial cells in the tears, and blood eosinophilia in some patients (Table 1).

In these 61 patients, 94 NPTs with inhalant allergens (Supplementary Table 2S), with respect to the positive skin tests and/or suspect disease history, and 61 phosphate-buffered saline (PBS) control tests were performed by means of rhinomanometry^{7–10,26,29,30} combined with the recording of ocular signs and symptoms.^{7–10,26,27} The patients were investigated in a period without acute ocular and nasal complaints and outside the allergen-relevant season. Long-acting H1-receptor antagonists and topical glucocorticosteroids were withdrawn 6 weeks before, whereas other treatments 48 h before each of the NPTs.

In these 61 patients, 61 NPTs with the allergens producing the SKCRs and 61 PBS controls (Supplementary Table 2S) were repeated 1–2 weeks later (Supplementary Figures 2S-I, II, III) and supplemented with cytologic examination of the tears. A 5-day interval was always inserted between the consecutive tests to prevent the carry-over effects. The study protocol was approved by the local ethical committee (IRB-MCK) and informed written consent was obtained from all study participants.

Statement of ethics

The author certifies that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research.

Allergens

Dialyzed and lyophilized allergen extracts (Allergopharma, Reinbek, Germany) were diluted in PBS and used for skin tests in concentrations of 100–500 BU/ml and for NPTs in concentrations of 1000–5000 BU/ml (Supplementary Table 2S), as recommended by the manufacturer. If indicated, higher allergen dilutions were used both for the skin tests and for the NPTs.

Skin tests, nasal provocation tests, control tests with phosphate-buffered saline, grading-scale and symptom-score system (Supplementary Table 1S), and survey of the allergens used for nasal challenge (Supplementary Table 2S) are presented in the on-line Supplementary Files.

Ocular (keratoconjunctival) response

The ocular features and subjective symptoms, registered before and during all NPTs with allergens and PBSs at the same time-points as the nasal NPG values, were assessed by ophthalmoscopy, including a slit lamp and vital staining with fluorescein. These parameters were evaluated by means of the modified Pelikan's grading system (Supplementary Table 1S).^{8–10,26,27}

Collection and processing of tears

The tears were collected from the fornix of each of the eyes separately by means of a micropipette before and repeatedly after the NPTs according to the following schedule: (a) SIKCR: before and 0–10, 20–30, 60, 90, 120, 180 and 240 min after the challenge (the tear samples collected from 0 to 10 and from 20 to 30 min were pooled with respect to the small tear amounts); (b) SLKCR and SDYKCR: before and 0, 30, 60, 90 and 120 min, every hour up to the 12th and every second hour during the time periods between the 24th–36th and 48th–56th hour after the challenge. The tear specimens were divided into three portions (each of ± 0.1 ml) and spread out on the slide surface using a glass probe. The first air-dried series was fixed by polyethylene glycol and stained by Hansel's method, the second air-dried series was stained by May-Grünwald-Giemsa, modified by us, and the third methanol fixed series was stained by the toluidine blue method.^{7,29–31} Specimens were dehydrated by methyl alcohol, mounted in Canada balsam and scanned microscopically.^{7,29–31} The absolute cell numbers were counted per microscopic field at magnification $\times 250$ and mean values were calculated from 20 fields for each eye separately. The mean values from both the eyes were finally expressed in number of cells per microscopic field ($\times 250$ magnification), per one eye. Doubtful cells were re-examined under oil immersion at magnification $\times 1200$.

The statistically significant magnitude of count changes (mean \pm SD) was as follows: eosinophils 2 (2.10 ± 0.14); neutrophils 2 (2.25 ± 0.21); basophils 0.3 (0.28 ± 0.04); mast cells 0.5 (0.35 ± 0.11); lymphocytes 1 (1.23 ± 0.16); monocytes 0.4 (0.41 ± 0.19); conjunctival epithelial (polygonal) cells 2 (2.17 ± 0.41); goblet cells 0.2 (0.20 ± 0.04); corneal epithelial cells (polygonal wing and/or flat superficial cells) 0.1 (0.12 ± 0.02).

Control group

In 15 allergic rhinitis patients, without ocular complaints and with normal ophthalmologic findings, volunteering to participate as control subjects, 15 positive NPTs were repeated and supplemented with recoding of the ocular features and cytologic examination of the tears.

Statistical analysis

The nasal and the ocular responses were evaluated by means of generalized multivariate analysis of variance model (MANOVA).³² The polynomials were fitted to the mean curves over time (30 time-points within 56 h after the allergen challenge) and the relevant hypotheses were tested by the modified MANOVA computerized system.

The mean NPG values and total keratoconjunctival scores of the same response type, compared with PBS control values at each of the time-points, were analyzed by the Mann-Whitney *U*-test. The changes of the cell counts during the NPTs and the PBS controls were analyzed by the Wilcoxon matched-pair signed rank test, comparing the post-challenge with the pre-challenge values at each of the time-points. Statistical evaluation of the keratoconjunctival responses was performed for each eye separately and then the mean from both the eyes was calculated and expressed for each eye. A *P* value < 0.05 was considered to be statistically significant.

Results

Nasal responses (NRs)

The 61 patients developed 20 immediate (INRs; $P < 0.001$), within 20–120 min after the challenge, 23 late (LNRs; $P < 0.001$), within 4–12 h after the challenge, 18 delayed (DYNRs; $P < 0.05$), within 26–56 h after the challenge, and 26 negative nasal responses (NNRs; $P > 0.05$). The repeated NPTs resulted in the development of similar INRs ($P < 0.01$), LNRs ($P < 0.001$), and DYNRs ($P < 0.01$) (Supplementary Figures 2S-I, II, III). The initial as well as the repeated PBS controls were all negative ($P < 0.05$, $P > 0.2$, respectively).

The particular nasal response types to allergen challenge (NRs) are presented in on-line supplement (S-4 and S-5).

SKCRs

The 61 repeated positive nasal responses induced 61 SKCRs (Table 1 and Supplementary Table 2S).

The immediate type (SIKCR; $n = 20$; $P < 0.01$, Figure 1b) appeared within 20 min, reached maximum between 30–45 min and resolved within 120 min after the NPT. The late type (SLKCR; $n = 23$; $P < 0.01$, Figure 1d) began

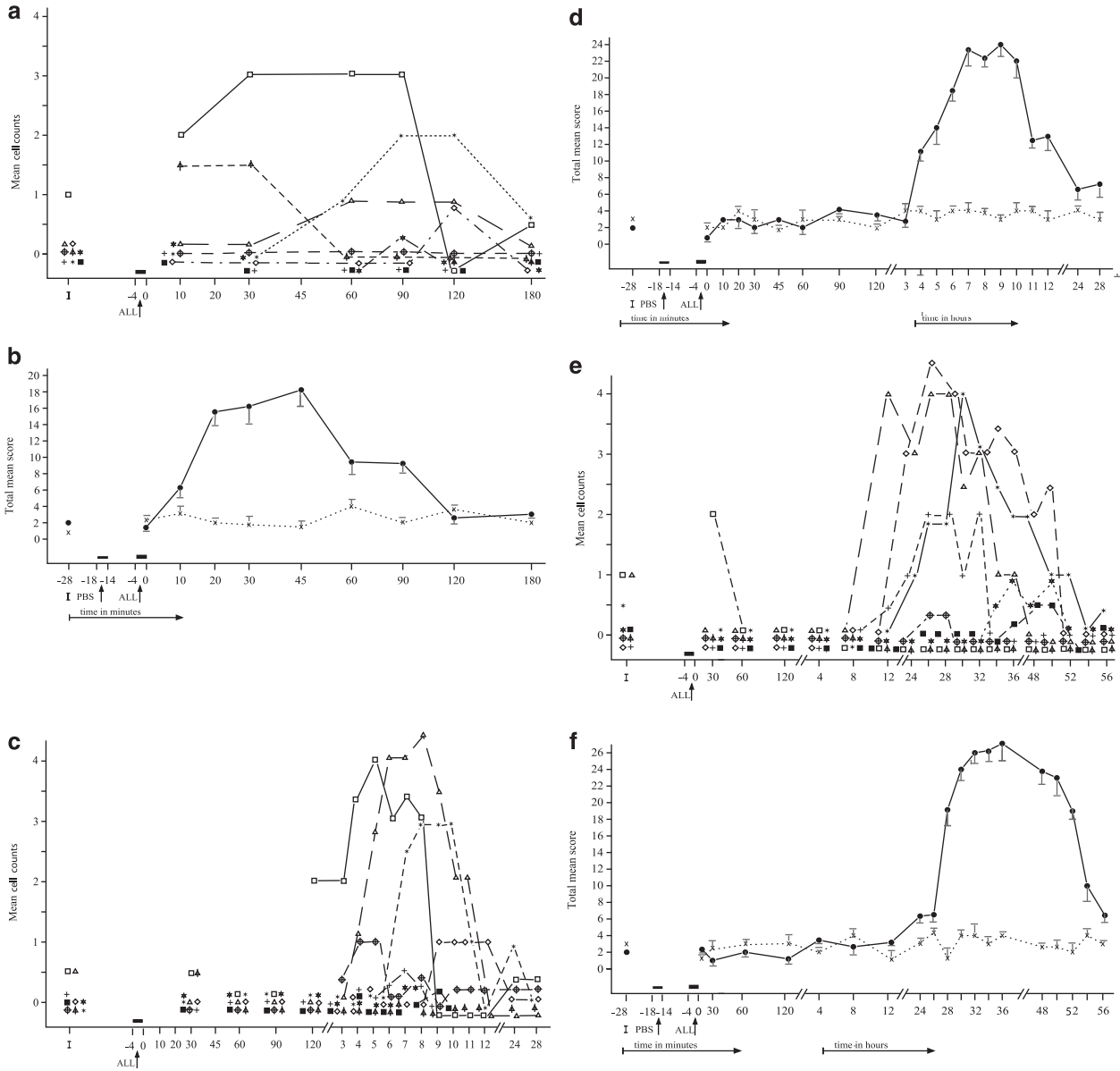


Figure 1 The secondary keratoconjunctival responses accompanying the nasal responses. The secondary immediate keratoconjunctival response (SIKCRs; $n = 20$). (a) The mean score of particular cell counts during the SIKCR: \square = eosinophils, Δ = neutrophils; \diamond = basophils, \blacktriangle = mast cells, \diamond = lymphocytes, $+$ = monocytes, \ast = goblet cells, \star = conjunctival epithelial cells, \blacksquare = corneal epithelial cells. (b) The mean total score of keratoconjunctival signs and symptoms during the SIKCR (\bullet) and PBS (\times). The secondary late keratoconjunctival responses (SLKCRs; $n = 23$). (c) The mean score of particular cell counts during the SLKCR: \square = eosinophils, Δ = neutrophils; \diamond = basophils, \blacktriangle = mast cells, \diamond = lymphocytes, $+$ = monocytes, \ast = goblet cells, \star = conjunctival epithelial cells, \blacksquare = corneal epithelial cells. (d) The mean total score of conjunctival signs and symptoms during the SLKCR (\bullet) and PBS (\times). The secondary delayed keratoconjunctival responses (SDYKCRs; $n = 18$). (e) The mean score of particular cell counts during the SDYKCR: \square = eosinophils, δ = neutrophils; \diamond = basophils, \blacktriangle = mast cells, \diamond = lymphocytes, $+$ = monocytes, \ast = goblet cells, \star = conjunctival epithelial cells, \blacksquare = corneal epithelial cells. (f) The mean total score of conjunctival signs and symptoms during the SDYKCR (\bullet) and PBS (\times). I = Initial (baseline) values, PBS = phosphate-buffered saline; ALL = allergen challenge. Bars represent mean \pm SEM.

between 4–5 h, reached maximum between 7–10 h and resolved within 12 after the challenge. The delayed type (SDYKCR; $n = 18$; $P < 0.05$, Figure 1f) appeared between 24–28 h, reached a maximum between 30–36 h and resolved within 56 h after the allergen challenge. In 16 VKC and 31 AKC patients an acute exacerbation of the atopic dermatitis, predominantly skin erythema and itching, were recorded during the positive SKCRs. No significant ocular signs were recorded during the 26 negative nasal responses ($P > 0.1$) or 57 PBS controls ($P > 0.2$). No significant differences in the ocular features were observed between the right and left eye ($P > 0.1$).

Cytologic changes in tears during the SKCRs

The SKCRs were accompanied by distinctly lower cell counts in tears as compared with counts found in tears during the primary keratoconjunctivitis types.^{8–10} The SIKCRs were associated with increased counts ($P < 0.05$) of mast cells between 10–30, eosinophils 20–90 and conjunctival epithelial cells between 90–120 min after the NPTs (Figure 1a; Table 2). The mast cells displayed wrinkling of cellular membrane, disappearance of intracellular granules, intracellular vacuolization, diminished stain intake and some of them were disrupted. The eosinophils were intact. The SLKCRs were accompanied by increased counts ($P < 0.05$) of eosinophils between 4–8 h, neutrophils between 5–9 h, basophils at 3–5 h and conjunctival epithelial cells

between 7–10 h, goblet cells between 7–8 h, and corneal epithelial cells at 9 h after the NPTs (Figure 1c; Table 3). The eosinophils, neutrophils, and basophils were intact. The conjunctival epithelial cells exhibited cellular deformation, cellular membrane wrinkling, and diminished stain intake. The SDYKCRs were associated with increased counts ($P < 0.05$) of neutrophils between 12–32 h, basophils between 26–28 h, lymphocytes between 24–50 h, monocytes between 12–32 h, conjunctival epithelial cells between 26–48 h, goblet cells between 34–50 h and corneal epithelial cells between 34–50 h after the NPTs (Figure 1e; Table 4). The neutrophils, lymphocytes, monocytes, and corneal epithelial cells were mostly intact. The conjunctival epithelial cells were deformed, with wrinkled cellular membrane, diminished cytoplasmic homogeneity and stain intake. The goblet cells were empty and wrinkled. During the 49 PBS controls and the 23 negative nasal responses only sporadic epithelial cells were observed. No significant differences in results were found between both the eyes ($P > 0.1$).

Control subjects

The 15 control subjects developed 5 immediate (INR; $P < 0.01$), 6 late (LNR, $P < 0.05$), and 4 delayed (DYNR; $P < 0.05$) nasal responses to repeated NPTs with allergen, without any accompanying ocular symptoms ($P > 0.1$). In the tears only sporadic intact epithelial cells (less than 1 cell per 5 microscopic fields) ($P > 0.2$) were recorded.

Discussion

The involvement of various hypersensitivity mechanisms results in different types of keratoconjunctival response, such as an immediate (IKCR), late (LKCR), or a delayed (DYKCR) type, each of them occurring either in a primary or in a secondary form, depending on the localization of the primary allergic reaction.^{3–10,12–16,18–22,25,33–36}

The conjunctiva has manifold anatomic and functional relationships with nasal mucosa, such as connection through the naso-lacrimal duct, facilitating the tear drainage into nasal cavity, and links through the blood vessel, lymphatic, and neurogenic network. These links allow the nasal allergic reaction to affect the conjunctiva in various ways upon the involvement of various mechanisms.^{3–5,7–10,12–22,29–31,37} Diagnostic confirmation of hypersensitivity mechanism(s) underlying the allergic keratoconjunctivitis consists usually of (1) an ophthalmologic examination, evaluation of the cornea by slit-lamp vital staining with fluorescein, and cytologic examination of the conjunctiva using brush, impression, or scraping techniques; (2) determination of specific

Table 2 Mean numbers of particular cell types in tears during the positive SIKCR and PBS control challenge ($n = 23$)

	Before the challenge	After the challenge (min)						
		0–10	20–30	60	90	120	180	240
<i>Eosinophils</i>								
SIKCR	1	2	3.0*	4.0*	3.0*	0	0	0.5
PBS	0.5	0	0.5	0	0	0.5	0	0.5
<i>Neutrophils</i>								
SIKCR	0	0	0	1	1	1	0	0
PBS	0	0	0	0	0	0	0	0
<i>Mast cells</i>								
SIKCR	0	1.5*	1.5*	0	0	0	0	0
PBS	0	0	0.5	0	0	0	0	0
<i>Conjunctival EC</i>								
SIKCR	0	0	0	1	2*	3*	0	0
PBS	0	0	0	0	0	0	0	0

Abbreviations: EC, epithelial cells; PBS, phosphate-buffered saline; SIKCR, secondary immediate keratoconjunctival response. Significance with respect to the baseline (before the challenge): * $P < 0.05$.

Table 3 Mean numbers of particular cell types in tears during the positive SLKCR and PBS control challenge ($n = 23$)

	Before the challenge	After the challenge (h)															
		½	1	1½	2	3	4	5	6	7	8	9	10	11	12	24	28
<i>Eosinophils</i>																	
SLKCR	0.5	0.5	0	0	2	2	4*	4*	3.5*	4*	3*	0	0	0	0	0.5	0.5
PBS	0.5	0	0	0	0	0	0.5	0	0	0	0	0	0	0	0	0.5	0
<i>Neutrophils</i>																	
SLKCR	0.5	0	0	0	0	0	1	3*	4*	4*	5*	4*	2	2	0	0	0
PBS	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0.5	0.5	0
<i>Basophils</i>																	
SLKCR	0	0	0	0.2	0	0.4*	1*	1.5*	0	0	0	0	0	0	0	0	0
PBS	0	0	0	0	0	0	0	0	0	0	0.3	0	0	0	0	0	0
<i>Conjunctival EC</i>																	
SLKCR	0.5	0	0	0	0	0	0	0	0	3*	3*	3*	3.2*	1	0	1	0
PBS	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1
<i>Goblet cells</i>																	
SLKCR	0	0	0	0	0	0	0	0	0.2	0.4*	0.5*	0	0	0	0	0	0
PBS	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0
<i>Corneal EC</i>																	
SLLCR	0	0	0	0	0	0	0	0	0	0	0	0.3*	0	0	0	0	0
PBS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Abbreviations: EC, epithelial cells; PBS, phosphate-buffered saline; SLKCR, secondary late keratoconjunctival response. Significance with respect to the baseline (before the challenge): * $P < 0.05$.

serum IgE antibodies and skin tests with allergens.^{1-6,23,28,33} These tests, generating static data, provide only general evidence for a possible existence of hypersensitivity mechanism elsewhere in the body, without further specification of its location and possible involvement of local antibodies in the particular organ.^{7-10,26,27}

Importance of the provocation tests

The only method matching the above-mentioned aspects are the provocation tests with allergens. These tests, performed on a certain organ, may definitely (1) confirm the existence of an allergic component in the particular and/or related organ(s); (2) demonstrate that a certain allergen causes indeed a certain response type in the particular organ, which can be measured quantitatively in its dynamic course; (3) to confirm the causal role of an allergic reaction occurring initially in one organ, in the secondarily induced response of another organ^{7,9,10,26,38-41}

The conjunctival provocation tests with allergens are a suitable technique to demonstrate the primary conjunctival/keratoconjunctival responses resulting from direct exposure of the ocular tissue to an external allergen.^{4,11,16,17,20,25,33,35,36,38,42-44} However, they are unable to detect conjunctival/keratoconjunctival responses induced secondarily by an allergic reaction

occurring initially in the nasal mucosa. These responses can only be detected by the NPT with allergens, in combination with registration of ocular features.^{7-10,26,40}

Cellular aspect of the allergic reaction

The allergy reaction in the conjunctival tissue, similar to the nasal mucosa, is a dynamic process caused by an external allergen in which various cell types are involved in the various steps of this process.^{3,4,6-22,28-31,34,37,43,44} This is also an exfoliative process leading to release of various cells into the particular media, such as tears or nasal secretions.^{1-11,13,16-22,25,26,28-30,34,36,44} These fluids serve therefore not only as conditioning means of the particular organs, but also as a means of drainage for released mediators and for removal of the exhausted, no longer active, cells having been eliminated by the mucosal tissues, after their active participation in the allergic reaction.^{7,10,27,40} The numbers and the condition of the expelled cells can indicate the qualitative and quantitative features of the allergic reaction in the particular mucosal membrane.^{7-10,16,23,26} However, the involvement of the individual cell types in the allergic reaction can only be evaluated by comparing their counts and their conditions before and repeatedly after a well-defined intervention, which is an allergen challenge.^{7-10,12,14,16,26,27,34,35,38}

Table 4 Mean numbers of particular cell types in tears during the positive SDYKCR and PBS control challenge (*n* = 18)

	Before the challenge	After the challenge (h)																	
		½	1	2	4	8	12	24	26	28	30	32	34	36	48	50	52	54	56
<i>Neutrophils</i>																			
SDYKCR	1	0	0	0	0	0	4*	4*	4*	4*	3*	3*	1	1	0	0	0	0	0
PBS	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
<i>Basophils</i>																			
SDYKCR	0	0	0	0	0	0	0	0.1	0.5*	0.3*	0	0.1	0	0	0	0	0	0	0
PBS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Lymphocytes</i>																			
SDYKCR	0	0	0	0	0	0	0	3*	5*	5*	4*	4*	4*	4*	3*	2.5*	0	0	0
PBS	0	0	0	0	0	0	0.5	0	0	0	1	0	0	0	0	0	0	1	0
<i>Monocytes</i>																			
SDYKCR	0	0	0	0	0	0	0.5*	1*	3*	2*	2.5*	2*	0	0	0	0	0.3	0	0
PBS	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0.2	0
<i>Conjunctival EC</i>																			
SDYKCR	0.5	0	0	0	0	0	0	1	2*	2*	4*	4*	2.5*	2.5*	2*	1	1	0	0.5
PBS	0.5	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0
<i>Goblet cells</i>																			
SDYKCR	0	0	0	0	0	0	0	0	0	0	0	0	0.5*	1*	1*	1*	0	0	0
PBS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Corneal EC</i>																			
SDYKCR	0	0	0	0	0	0	0	0	0	0	0	0.1	0.2*	0.6*	0.6*	0.5*	0	0	0
PBS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Abbreviations: EC, epithelial cells; PBS, phosphate-buffered saline; SDYKCR, secondary delayed keratoconjunctival response. Significance with respect to the baseline (before the challenge): **P* < 0.05.

Cytologic examination of the tears and conjunctiva

The cytologic examination of the conjunctiva can be performed by semi-invasive techniques, such as brush or impression technique, or by invasive techniques, such as scraping or classical biopsy.^{13,16–22,28,34} These techniques generate data concerning the cellular aspects of allergy mechanism(s) in the epithelial and sub-epithelial layers. However, their disadvantages, except the brush method, are the use of anesthetics, some traumatizing effects of the ocular tissues, and stimulation of the neurogenic receptors causing undesirable reflexes. Moreover, these techniques cannot be repeated on the same conjunctival sites owing to traumatizing effects on the mucosal capillaries and possible disturbance of the tissue repair. *Vice versa*, the results attained from different conjunctival and/or corneal localities are not fully comparable.⁷ The cytological data generated by these techniques demonstrated some variations in presence of particular cell types in the conjunctival tissue. Mostly eosinophils and neutrophils, sporadically mast cells and lymphocytes, were found in conjunctival epithelium and sub-epithelial layers.^{11,13,16,19–22,25,28,34–36} However,

because of their unsuitability for repeated application on the same location, no dynamic course of the cellular changes in the conjunctival tissue could be gained by these techniques. On the other hand, the cytologic examination of tears, collected by means of aspiration with a micropipette, is a very simple and valuable method, which can be repeated almost endlessly, requires no anesthesia, does not traumatize the ocular tissue, and is most similar to the natural drainage of tears.^{7,10,13,17,20,23,25,27,34} However, results produced by this technique are valid only for cellular events in tears and not in the mucosal membrane. This may only be derived from a mucosal tissue biopsy.^{13,14,17,20–22,34,36}

The cytologic examination of the tears is customarily performed by means of a single-tear cytogram, revealing increased numbers of eosinophils, mast cells, and conjunctival epithelial cells and sometimes neutrophils. Nevertheless, the cytologic studies of tears in keratoconjunctivitis patients are not numerous.^{11,16,20,25,36,43} There is a dearth of data documenting the dynamic course of the cytologic changes in tears during particular types of the primary keratoconjunctival response to CPT with allergens.^{2,3,10,11,16,20,25,34,36,44} Moreover, no

information concerning the cytologic changes in the tears during the SKCRs induced by the nasal allergy are available. Our findings of relatively lower counts of all cell types, together with intact granulocytes and mononuclear cells in tears during the SKCRs, would suggest their non-participation in the SKCRs, before their migration from the conjunctival capillaries into the tears. These findings are in contrast to the higher cell counts in tears during the primary conjunctival or keratoconjunctival responses^{7,8,10,17,29–31,35} as well as to the high counts of the same cell lineages in the NS.^{7,10,29–31}

The increased counts of corneal epithelial cells in tears during the SDYKCR indicate the involvement of cornea in this response type. The cytologic profiles in tears during the particular SKCR types showed partly similar patterns to the cytologic changes found by us in tears during the secondary conjunctival response types (SCRs).²⁷ However, the SKCRs differ from the SCR types not only in slightly higher numbers of all cells in tears, but also in course of count changes of individual cell types and finally in appearance of corneal epithelial cells. These differences would indicate larger and more serious immunologic mechanisms underlying the SKCRs than those involved in SCR types. Regarding our not yet published data, the differences in the cytological findings in tears between the SKCRs and SCR types may probably be explained by different participation of particular factors, such as mediators and cytokines, released by the primary allergic reaction in the nasal mucosa before they reached conjunctivae. Nevertheless, the exact route by which the mediators, cytokines, and other factors released in the nasal mucosa may reach the conjunctivae, and subsequently also cornea, as well as the mode of their action, is not yet fully clarified and will need more concurrent studies.

Summary

What was known before

- The secondary keratoconjunctival response types induced by nasal allergy have first been reported by the author in 2009.⁹
- There is a dearth of information and knowledge concerning this topic in the literature.

What this study adds

- This study contributes to (1) better understanding of the pathophysiological mechanisms underlying the secondary keratoconjunctival response types; (2) the differentiation of the particular KC response types; (3) the involvement of particular cell lineages in the KC response types; (4) demonstration of the diagnostic value of nasal allergen challenge combined with recording of the ocular features and cytologic profiles in the tears in some KC patients.

Conflict of interest

The authors declare no conflict of interest.

References

- 1 Barney NP, Graziano FM. Allergic and immunologic diseases of the eye. In: Adkinson NF, Yunginger JW, Busse WW, Bochner BS, Holgate ST, Simons FER (eds) *Middleton's Allergy, Principles & Practice*, 6th edn. Mosby-Elsevier Inc.: Philadelphia, PA, USA, 2003, pp 1599–1617.
- 2 Bielory L, Friedlaender MH. Allergic conjunctivitis. *Immunol Allergy Clin N Am* 2008; **28**: 43–57.
- 3 Hingorani M, Lightman S. Ocular allergy. In: Kay AB ed. *Allergy and Allergic Diseases*. Blackwell Science: Oxford, UK, 1997, pp 1645–1670.
- 4 Bielory L. Allergic and immunologic disorders of the eye; Part II: Ocular allergy. *J Allergy Clin Immunol* 2000; **106**: 1019–1032.
- 5 Barney NP, Graziano FM, Cook EB, Stahl JL. Allergic and immunologic diseases of the eye. In: Adkinson NF, Bochner BS, Busse WW, Holgate ST, Lemanske RF, Simons FE (eds). *Middleton's Allergy, Principles & Practice*, 7th edn. Mosby-Elsevier Inc: Philadelphia, PA, USA, 2009, pp 1117–1137.
- 6 Gurbaxani A, Calder VL, Lightman S. Ocular allergy. In: Kay AB, Kaplan AP, Bousquet J, Holt P (eds). *Allergy and Allergic Diseases*, 2nd edn. Wiley Blackwell Publ Ltd: Oxford, UK, 2000, pp 1482–1509.
- 7 Pelikan Z. The late nasal response. *Thesis*. The Free University of Amsterdam: Amsterdam, 1996.
- 8 Pelikan Z. The causal role of the nasal allergy in some patients with allergic conjunctivitis. *Allergy* 2002; **57**(Suppl 73): 230.
- 9 Pelikan Z. The possible involvement of nasal allergy in allergic keratoconjunctivitis. *Eye* 2009; **23**: 1653–1660.
- 10 Pelikan Z. Allergic conjunctivitis and nasal allergy. *Curr Allergy Asthma Rep* 2010; **10**: 295–302.
- 11 Bonini S, Bonini S, Bucci MG, Berruto A, Adriani E, Balsano F *et al*. Allergen dose response and late symptoms in a human model of ocular allergy. *J Allergy Clin Immunol* 1990; **86**: 869–876.
- 12 Bonini S, Lambiase A, Sacchetti M, Bonini S. Cytokines in ocular allergy. *Int Ophthalmol Clin* 2003; **43**: 27–32.
- 13 Calder VL. Cellular mechanisms of chronic cell-mediated allergic conjunctivitis. *Clin Exp Allergy* 2002; **32**: 814–817.
- 14 Choi SH, Bielory L. Late-phase reaction in ocular allergy. *Curr Opin Allergy Clin Immunol* 2008; **8**: 438–444.
- 15 Cook EB. Tear cytokines in acute and chronic ocular allergic inflammation. *Curr Opin Allergy Clin Immunol* 2004; **4**: 441–445.
- 16 Leonardi A. Vernal keratoconjunctivitis: pathogenesis and treatment. *Prog Retin Eye Res* 2002; **21**: 319–339.
- 17 Leonardi A. In-vivo diagnostic measurements of ocular inflammation. *Curr Opin Allergy Clin Immunol* 2005; **5**: 464–472.
- 18 Leonardi A, Curnow SJ, Zhan H, Calder VL. Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. *Clin Exp Allergy* 2006; **36**: 777–784.
- 19 Leonardi A, Fregona IA, Plebani M, Secchi AG, Calder VL. Th1- and Th2-type cytokines in chronic ocular allergy. *Graefe's Arch Clin Exp Ophthalmol* 2006; **244**: 1240–1245.

- 20 Leonardi A, De Dominics C, Motterle L. Immunopathogenesis of ocular allergy: a schematic approach to different clinical entities. *Curr Opin Allergy Clin Immunol* 2007; **7**: 429–435.
- 21 Metz DP, Bacon AS, Holgate ST, Lightman SL. Phenotypic characterization of T cells infiltrating the conjunctiva in chronic allergic eye disease. *J Allergy Clin Immunol* 1996; **98**: 686–696.
- 22 Metz DP, Hingorani M, Calder VL, Buckley RJ, Lightman SL. T-cell cytokines in chronic allergic eye disease. *J Allergy Clin Immunol* 1997; **100**: 817–824.
- 23 Ono SA, Abelson MB. Allergic conjunctivitis: update on pathophysiology and prospects for future treatment. *J Allergy Clin Immunol* 2005; **115**: 118–122.
- 24 Stern ME, Siemasko KF, Niederkorn JY. The Th1/Th2 paradigm in ocular allergy. *Curr Opin Allergy Clin Immunol* 2005; **5**: 446–450.
- 25 Uchio E, Ono SY, Ikezawa Z, Ohno S. Tear levels of interferon-gamma, interleukin (IL)-2, IL-4 and IL-5 in patients with vernal keratoconjunctivitis, atopic keratoconjunctivitis and allergic conjunctivitis. *Clin Exp Allergy* 2000; **30**: 103–109.
- 26 Pelikan Z. Seasonal and perennial allergic conjunctivitis: the possible role of nasal allergy. *Clin Exp Ophthalmol* 2009; **37**: 448–457.
- 27 Pelikan Z. Cytologic changes in tears during the secondary conjunctival response induced by nasal allergy. *Br J Ophthalmol* 2012; **96**: 941–948.
- 28 Tsubota K, Takamura E, Hasegawa T, Kobayashi T. Detection by brush cytology of mast cells and eosinophils in allergic and vernal conjunctivitis. *Cornea* 1991; **10**: 525–531.
- 29 Pelikan Z, Pelikan-Filipek M. Cytologic changes in the nasal secretions during the immediate nasal response. *J Allergy Clin Immunol* 1988; **82**: 1103–1112.
- 30 Pelikan Z, Pelikan-Filipek M. Cytologic changes in the nasal secretions during the late nasal response. *J Allergy Clin Immunol* 1989; **83**: 1068–1079.
- 31 Pelikan Z, Pelikan-Filipek M. Intracellular changes in some cell types in nasal secretions (NS) during the late nasal response (LNR) to allergen challenge (NPT). *Clin Exp Allergy* 1990; **20**(Suppl to No 1): 60(Abstr P 131).
- 32 Tabachnick BG, Fidell LS. *Using Multivariate Statistics*. Harper Collins College Publishers: New York, 1996.
- 33 Anderson DF. The conjunctival late-phase reaction and allergen provocation in the eye. *Clin Exp Allergy* 1996; **26**: 1105–1107.
- 34 Bacon AS, Ahluwalia P, Irani AM, Schwartz LB, Holgate ST, Church MK *et al*. Tear and conjunctival changes during the allergen-induced early- and late-phase responses. *J Allergy Clin Immunol* 2000; **106**: 948–954.
- 35 Bonini S, Bonini S, Berruto A, Tomassini M, Carlesimo S, Bucci MG *et al*. Conjunctival provocation test as a model for the study of allergy and inflammation in humans. *Int Arch Allergy Appl Immunol* 1989; **88**: 144–148.
- 36 Friedlaender MH. Conjunctival provocative tests: a model of human ocular allergy. *Tr Am Ophthalmol Soc* 1989; **87**: 577–597.
- 37 Dua HS, Gomes JA, Donoso LA, Leibson PR. The ocular surface as part of the mucosal immune system: conjunctival mucosa-specific lymphocytes in ocular surface pathology. *Eye* 1995; **9**: 261–267.
- 38 Melillo G, Bonini S, Cocco G, Davies RJ, Dr Monchy JGR, Frolund L *et al*. Provocation tests with allergens. *Allergy* 1997; **52**(Suppl 35): 5–36.
- 39 Takano Y, Fukagawa K, Dogru M, Asano-Kato N, Tsubota K, Fujishima H. Inflammatory cells in brush cytology samples correlate with severity of corneal lesions in atopic keratoconjunctivitis. *Br J Ophthalmol* 2004; **88**: 1504–1505.
- 40 Naclerio RM, Norman PS, Fish JE. In vivo methods for the study of allergy-mucosal tests, techniques, and interpretations. In: Middleton E, Reed CE, Ellis EF, Adkinson NF, Yunginger JW, Busse WW (eds) *Allergy, Principles and Practice*, 4th edn. Mosby-Year Book, Inc: St. Louis, MI, USA, 1993, pp 595–637.
- 41 Pelikan Z. Provocation tests (PT). I. Definition, reason, principles, indications, contra-indications, conditions, requirements, contribution to the diagnostic procedures and therapeutic management. *Alergie (Cz)* 2007; **9**: 32–38.
- 42 O'Sullivan NL, Montgomery PC, Sullivan DA. Ocular mucosal immunity. In: Mestecky J, Bienenstock J, Lamm M, Strober W, McGhee J, Mayer L (eds) *Mucosal Immunology*, 3rd edn Elsevier- Academic Press: Burlington, MA, USA; San Diego, CA, USA; London, UK, 2005, pp 477–1496.
- 43 Abelson MB, Chambers WA, Smith LM. Conjunctival allergen challenge. A clinical approach to studying allergic conjunctivitis. *Arch Ophthalmol* 1990; **108**: 84–88.
- 44 Helintö M, Renkonen R, Tervo T, Vesaluoma M, Saaren-Seppala H, Haahtela T *et al*. Direct in vivo monitoring of acute allergic reactions in human conjunctiva. *J Immunol* 2004; **172**: 3235–3242.

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