

Cytokines in Tears during the Secondary Keratoconjunctival Responses Induced by Allergic Reaction in the Nasal Mucosa

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Key Words

Allergic keratoconjunctivitis · Secondary keratoconjunctival responses · Nasal allergy · Nasal provocation tests with allergens · Cytokines in tears

Abstract

Background: Allergic keratoconjunctivitis (KC) can occur in a primary form due to an allergic reaction taking place in the conjunctivae or in a secondary form induced by nasal allergy.

Objectives: To search for the cytokine changes in tears accompanying the secondary keratoconjunctival response types (SKCR), caused by the nasal allergy. **Methods:** In 43 KC patients developing 15 immediate (SIKCR), 16 late (SLKCR) and 12 delayed (SDYKCR) responses to nasal provocation tests with allergens (NPT), the NPTs were repeated with subsequent recording of cytokine concentrations in tears up to 72 h. **Results:** The SIKCRs ($p < 0.001$), occurring 10–120 min after the NPT, were accompanied by significant changes ($p < 0.05$) of interleukin (IL)-4, IL-6, IL-10, IL-12p70 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The SLKCRs ($p < 0.01$), appearing 5–12 h after the NPT, were associated with significant changes ($p < 0.05$) of IL-3, IL-4, IL-5, IL-8, IL-10, tumor necrosis factor (TNF)- α , GM-CSF and granulocyte colony-stimulating factor. The SDYKCRs ($p < 0.01$), occurring 24–48 h after the NPT, were accompanied by significant changes ($p < 0.05$) of IL-2, IL-8, IL-10, interferon- γ , transforming growth factor- β and TNF- α . **Conclusions:** The particular SKCR types, induced by an allergic reaction in the

nasal mucosa, were accompanied by different cytokine profiles in the tears, suggesting involvement of different hypersensitivity mechanisms. These results also stress the diagnostic usefulness of NPTs combined with monitoring of ocular features in KC patients who did not respond satisfactorily to the topical ophthalmological treatment.

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Introduction

Allergic keratoconjunctivitis (KC), disorders in which an allergic component plays a principal role and in which both the conjunctiva and the cornea are affected, can be divided into: (a) the pollen-related ‘vernal keratoconjunctivitis’ (VKC) and (b) the perennial allergen-related ‘atopic keratoconjunctivitis’ (AKC) [1–3].

With respect to the location of the allergic reaction, 2 AKC forms can be distinguished; a primary AKC form, where the allergic reaction is localized in the conjunctiva, and a secondary AKC form, whereby the allergic reaction occurring primarily in the nasal mucosa induces a secondary keratoconjunctival response (SKCR) [4–7].

The involvement of different hypersensitivity mechanisms, such as immediate [immunoglobulin E (IgE)-mediated, type I], late (type III) or delayed (cell-mediated, type IV) types in the primary as well as in the secondary AKC form, results in the development of different types of the primary as well as secondary kera-

toconjunctival response (KCR), e.g. an immediate/early (IKCR), late (LKCR) or delayed (DYKCR) response [2, 4–18].

The cytokines in the tears of the KC patients have already been studied from various points of view [1, 5, 6, 14–17, 19–36]. Nevertheless, there is a dearth of data related to the course of the cytokine changes in tears during the individual KCR types of the primary KC form [1–3, 9–13, 15, 17, 19, 20, 37–43]. Moreover, no data are available to illustrate the concentration changes of the cytokines in tears accompanying the particular SKCR types [14].

The purpose of this study was: (1) to search the cytokine changes during the individual types of the SKCR; (2) to assess the possible activation of various cell types during the particular SKCR types with respect to the cytokine profiles in tears; (3) to evaluate the significance of the cytokine concentration changes in tears for the involvement of particular hypersensitivity mechanisms in the individual SKCR types.

Methods

Patients

Some patients suffering from allergic KC and responding unsatisfactorily to the usual topical ophthalmological therapy were referred to our Department of Allergology and Immunology (Institute of Medical Sciences De Klokkenberg, Breda, the Netherlands) during 1998–1999 for more extensive diagnostic analysis of their ocular complaints. The 43 KC patients (19 VKC and 24 AKC) developing a KCR after the nasal challenge with allergen, during the routine diagnostic procedure, have volunteered to participate in this study.

These 43 patients, 20 men and 23 women, 18–42 years of age (table 1), had been treated with topical as well as oral H₁ receptor antagonists, topical glucocorticosteroids and decongestant in the past, however without significant therapeutic effects. In 37 of these patients (16 VKC and 21 AKC), an atopic dermatitis on the forehead, in the periorbital area or on the face was found, and 2 of these patients suffered also from bronchial asthma. All 43 patients demonstrated normal intraocular pressure. These patients underwent a routine diagnostic procedure (table 1), and ophthalmological examination including ophthalmoscopy, slitlamp evaluation, vital staining with fluorescein and cytological screening of the tears.

The routine diagnostic procedure revealed a positive or suspected history for nasal allergy (98%), hyperemic conjunctivae (100%), conjunctival epithelial cells and sporadic eosinophils in the tears (86%), edematous nasal mucosa (95%), positive skin tests with inhalant allergens (100%), increased blood eosinophilia (25%) and positive specific IgE in the serum for some inhalant allergens (18%; table 1).

In these 43 patients, 101 nasal provocation tests (NPTs) with inhalant allergens (online suppl. table 2S; for all online suppl. material, see www.karger.com/doi/10.1159/000358200) and 43 con-

trol challenges with phosphate-buffered saline (PBS) were performed by means of rhinomanometry [4–7, 14, 21] in combination with simultaneous recording of the ocular features and subjective symptoms. The patients were investigated in a period without acute ocular and nasal complaints, outside the allergen-relevant season and during a short hospitalization.

The long-acting H₁ receptor antagonists (n = 17), topical cromolyn (n = 9) and glucocorticosteroids (n = 11) were withdrawn 4 weeks and the other treatments were discontinued 48 h before the study. The 43 positive NPTs producing an SKCR of any type and 43 PBS controls were repeated 3 weeks later and supplemented with collection of the tear samples for the cytokine determination. A 5-day interval was always inserted between the subsequent tests to prevent the carryover effects. The study protocol was approved by the local ethical committee (IRB-MCK), and a written informed consent was obtained from all study participants. The study was carried out according to the Declaration of Helsinki concerning the 'Principles for medical research involving the human subjects'.

Routine diagnostic procedure, allergens (see online suppl. table 1S), skin tests, NPTs (with allergens and control challenges with PBS), grading scale and symptom score system (see online suppl. table 2S), cytokine immunoassay kits and additional statistical analysis are presented in the online supplementary files.

Ocular KCR

The objective conjunctival and corneal signs and subjective symptoms were registered before and during all NPTs with allergens and PBS controls at the same time points as the nasal NPT values. The features of the conjunctiva and cornea were assessed by ophthalmoscopy including a slitlamp examination and vital staining with fluorescein.

The conjunctival signs (hyperemia/injection, chemosis, hyperlacrimation, papillary hypertrophy, cobblestoning and palpebral edema), the corneal signs (diminished corneal sheen, transparency and reflexion, scars, appearance of Horner-Tranta dots and epithelial defects) and the subjective symptoms (itching/burning, blurred and/or disturbed vision, foreign body feeling, ocular pain and photophobia) were registered and evaluated by means of the Pelikan scale (online suppl. table 2S) [5–7, 14].

Collection and Processing of the Tears

The tears were collected from the inferior conjunctival fornix and/or lacus lacrimalis of each of the eyes separately by a micropipette, at the following time intervals: (a) before the NPT, 1 mixed sample of tears collected at 10, 5 and 0 min; (b) after the NPT, a mixed tear sample collected at 0–10 min and at 20–30 min, and (c) separate tear samples collected at 45, 60, 120 and 240 min, and every second hour up to 12 h and between the 24–38th and 48–56th (60th) hours. The tear samples (70–150 µl) were centrifuged at 2,000 g at 4°C for 5 min, and supernatants were stored at –20°C and processed within 12 h.

Determination of Cytokines in Tears

The cytokines were determined in supernatants by commercial enzyme-linked immunosorbent assay kits, according to the manufacturer's recommendations. Tear samples from each eye were measured separately on each occasion, applying a double-blind schedule, in duplicate. The results were calculated as the mean of both eyes.

Table 1. Characteristics of the patients with allergic KC

	Patients (n = 43)			Control subjects (n = 18)
	SIKCR (n = 15)	SLKCR (n = 16)	SDYKCR (n = 12)	
Age, years	21±3	25±6	22±5	27±4
Sex (M/F)	6/9	7/9	7/5	8/10
Disease history, years	3.1±0.7	3.5±1.5	3.4±1.2	0
Blood leukocyte count ^a , n × 10 ⁹ /l	7.1±0.9	7.7±1.0	8.2±0.6	8.0±1.2
Blood eosinophil count ^b , n × 10 ⁶ /l	345±28	323±25	267±14	265±17
Increased total IgE in the serum ^c	0	0	0	0
Positive specific IgE in the serum ^d	5	3	0	1
Positive skin response ^e				
Immediate type	10	7	1	8
Late type	5	9	2	7
Delayed type	0	0	9	3
Nasal histamine threshold ^f				
Decreased	9	7	1	8
Normal	6	9	11	10
Nasal provocation tests				
Positive	15	16	12	18
Negative	19	22	17	10
Cytokines in tears, pg/ml				
IL-1β	<1.0	<1.0	<1.0	<1.0
IL-2	<3.0	<3.0	3.2±0.1	<3.0
IL-3	<31.2	<31.2	<31.2	<31.2
IL-4	<0.6	<0.6	<0.6	<0.6
IL-5	<3.0	3.4±0.3	<3.0	<3.0
IL-6	<4.0	<4.0	<4.0	<4.0
IL-7	<0.1	<0.1	<0.1	<0.1
IL-8	<1.3	2.1±0.6	2.4±1.0	<1.3
IL-10	<3.0	3.4±0.2	<3.0	<3.0
IL-12p40	<3.9	<3.9	<3.9	<3.9
IL-12p70	2.2±0.1	<2.1	<2.1	<2.1
IL-13	<3.0	<3.0	<3.0	<3.0
IL-18	<9.2	<9.2	<9.2	<9.2
IFN-γ	<1.0	<1.0	1.2±0.1	<1.0
GM-CSF	3.6±0.5	<3.0	<3.0	<3.0
G-CSF	<0.8	1.5±0.4	<0.8	<0.8
TNF-α	<6.0	6.7±0.5	<6.0	<6.0
TGF-β	6.3±0.2	<6.0	<6.0	<6.0

SIKCR = Secondary immediate keratoconjunctival response; SLKCR = secondary late keratoconjunctival response; SDYKCR = secondary delayed keratoconjunctival response; M = male; F = female; IgE = immunoglobulin E; IL = interleukin; IFN-γ = interferon-γ; GM-CSF = granulocyte-macrophage colony-stimulating factor; G-CSF = granulocyte colony-stimulating factor; TNF-α = tumor necrosis factor-α; TGF-β = transforming growth factor-β. Values are means ± SEM or numbers. The detection limits of cytokines (pg/ml): IL-1β, 1.0; IL-2, <3.0; IL-3, 31.2; IL-4, 0.6; IL-5, 3.0; IL-6, 4.0; IL-7, 0.1; IL-8, 1.3; IL-10, <3.0; IL-12p40, 3.9; IL-12p70, 2.1; IL-13, <3.0; IL-18, 9.2; IFN-γ, 1.0; GM-CSF, <3.0; G-CSF, 0.8; TNF-α, 6.0; TGF-β, 6.0.

^a Normal value 4.0–10.0 × 10⁹/l.

^b Normal value <300 × 10⁶/l.

^c Normal value <500 IU/ml.

^d Normal value <0.70 U/ml.

^e Positive skin response to the relevant allergen (=allergen-producing positive nasal and keratoconjunctival or nasal response only).

^f Normal value >8.0 mg/ml.

Control Group

In 18 patients with allergic rhinitis without ocular complaints and with normal ophthalmological findings volunteering to participate as control subjects, 18 positive NPTs with inhalant allergens (6 immediate, 7 late, 5 delayed nasal responses) were repeated and supplemented with recording of the ocular and subjective symptoms and single prechallenge determination of the cytokines in tears.

Statistical Analysis

The postchallenge cytokine values recorded at each of the time points during the repeated NPT and PBS controls in each of the patients were compared with their prechallenge values and analyzed by the Wilcoxon matched-pair signed rank test. The mean postchallenge cytokine values measured at each of the time points during the repeated NPTs were also compared with corresponding PBS values and evaluated by the Mann-Whitney U test. Statistical evaluation was performed separately for each eye, and the mean of both eyes was then calculated. A p value <0.05 was considered to be statistically significant.

Results

The nasal response types are presented in figures 1c, 2c, 3c and in the online supplementary material.

Ocular KCRs

The 43 positive nasal responses were associated with significantly positive SKCRs, i.e. 15 SIKCR ($p < 0.001$), 16 SLKCR ($p < 0.01$) and 12 SDYKCR ($p < 0.01$). No SKCRs were observed during the 58 negative nasal responses ($p > 0.2$). The SKCRs were characterized by significant changes of the objective conjunctival signs ($p < 0.01$) and corneal signs ($p < 0.01$) and subjective symptoms ($p < 0.05$). The 43 repeated NPTs induced similar and statistically significant SKCRs, both as comparing the postchallenge with the prechallenge (baseline) values ($p < 0.01$) and as comparing with the PBS control challenge ($p < 0.01$; fig. 1b, 2b, 3b). No significant differences in the ocular features were recorded between the right and left eye during the initial and the repeated SKCRs ($p > 0.1$). No ocular symptoms were recorded during the 43 PBS control challenges ($p > 0.1$) or during the 58 negative KCRs ($p > 0.1$).

Changes of the Cytokine Concentrations in Tears during the SKCRs

The SIKCRs were accompanied by significant concentration changes ($p < 0.05$) of interleukin (IL)-4, IL-6, IL-10, IL-12p70 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the tears (table 2; fig. 1a), whereas the concentrations of IL-1 β , IL-2, IL-3, IL-5, IL-

7, IL-8, IL-12p40, IL-13, IL-18, interferon (IFN)- γ , granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor (TNF)- α and transforming growth factor (TGF)- β in tears were either under the detection limits or unchanged. The SLKCRs were associated with significant changes in the concentrations ($p < 0.05$) of IL-3, IL-4, IL-5, IL-8, IL-10, GM-CSF, G-CSF and TNF- α (table 3; fig. 2a), whereas the concentrations of IL-1 β , IL-2, IL-7, IL-12p40, IL-12p70, IL-13, IL-18, IFN- γ and TGF- β remained unchanged or under the detection limits. The SDYKCRs were accompanied by significant concentration changes ($p < 0.05$) of IL-2, IL-8, IL-10, IFN- γ , TNF- α and TGF- β in the tears (table 4; fig. 3a), whereas the concentrations of other cytokines, such as IL-1 β , IL-3, IL-4, IL-5, IL-6, IL-7, IL-12p40, IL-12p70, IL-13, IL-18, GM-CSF and G-CSF were either unchanged or under the detection limits. The prechallenge concentrations of most of the cytokines in tears were relatively low or under the detection limit and did not differ significantly from values measured in the healthy control subjects (table 1). No significant concentration changes of these cytokines were recorded in tears during the 43 PBS controls ($p > 0.1$) or 58 negative KCRs ($p > 0.1$). No significant differences in the cytokine concentration changes were found in tears between the right and left eye, either during the SKCRs or during the PBS controls ($p > 0.1$ and $p > 0.2$, respectively).

Control Patients

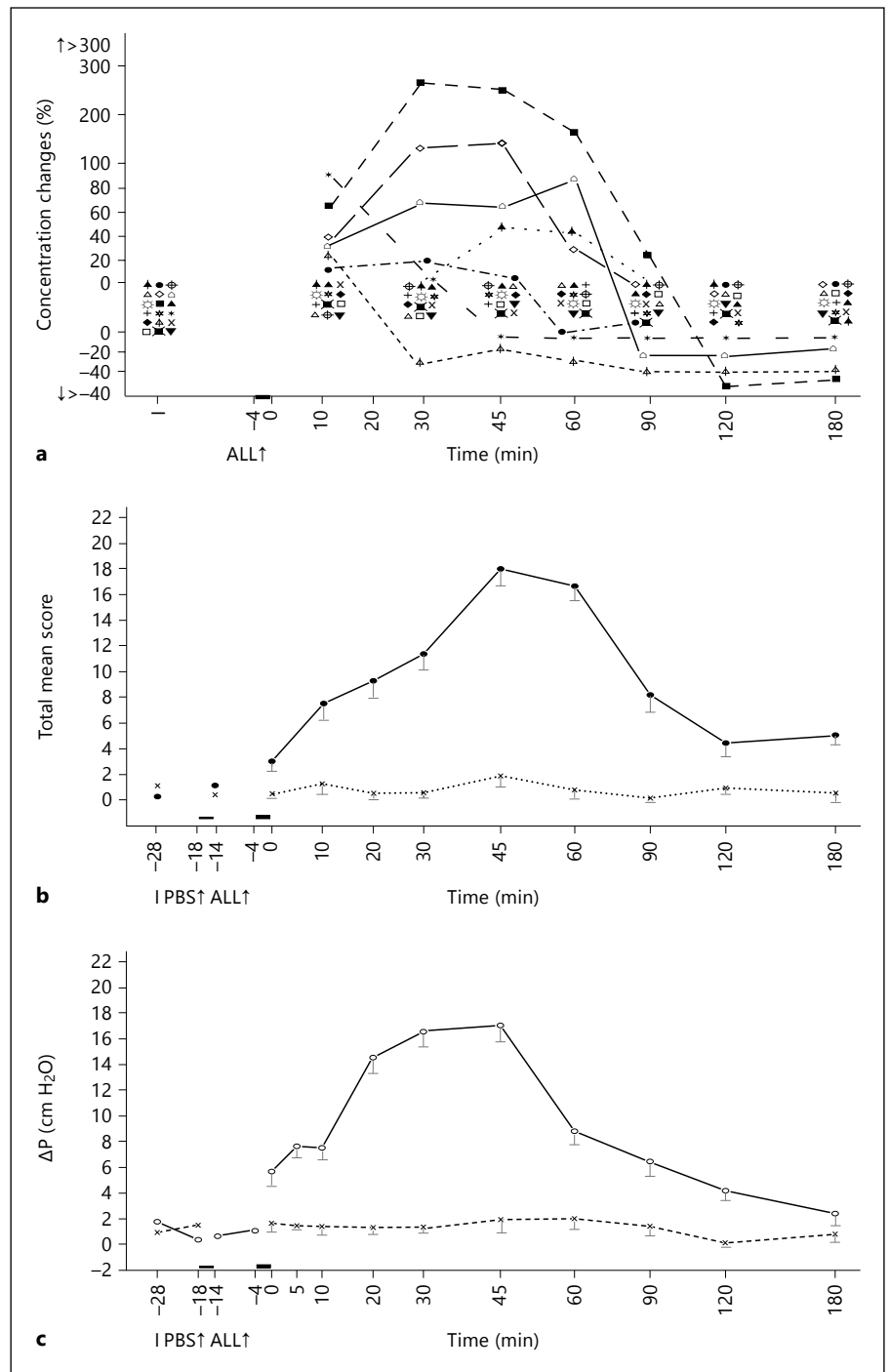
The 18 control subjects did not demonstrate any ocular signs or subjective symptoms during the 18 repeated positive nasal responses. Their prechallenge cytokine concentrations in tears are presented in table 1.

Discussion

The existence of the primary AKC form due to the initial allergic reaction occurring in the conjunctivae, and the secondary AKC form, induced by the primary allergic reaction taking place in the nasal mucosa, may have a significant impact both on the diagnostic procedure and on the treatment of this disorder [1–7, 14].

The manifold relationships between the conjunctivae and the nasal cavity, realized both at the anatomical and the functional level [1–7, 14, 18, 19, 41], include the following components: (a) the nasolacrimal duct, connecting the conjunctivae with the nasal cavity and executing the tear drainage into the nasal cavity and under certain conditions allowing also a retrograde penetration of the cells and various factors, released by and in the nasal mu-

Fig. 1. The SIKCRs (n = 15). I = Initial (baseline) values; ALL = allergen. **a** The mean score of particular factors during the SIKCR. Φ = IL-1 β ; $\square$ = IL-2; Δ = IL-3; $\blacksquare$ = IL-4; $\blacktriangle$ = IL-5; $\blacktriangleup$ = IL-6; $\star$ = IL-7; $\blacktriangleleft$ = IL-8; $\diamond$ = IL-10; $\blacksquare$ = IL-12p40; $\star$ = IL-12p70; $\diamond$ = IL-13; $+$ = IL-18; $\blacklozenge$ = IFN- γ ; $\triangle$ = GM-CSF; x = G-CSF; $\blacktriangledown$ = TNF- α ; $\bullet$ = TGF- β . **b** The total mean score of keratoconjunctival signs and symptoms during the SIKCR ($\bullet$) and PBS control (x). **c** The mean rhinomanometric values (nasopharynx-nostril pressure gradients) recorded during the immediate nasal response ($\circ$) and PBS control (x).

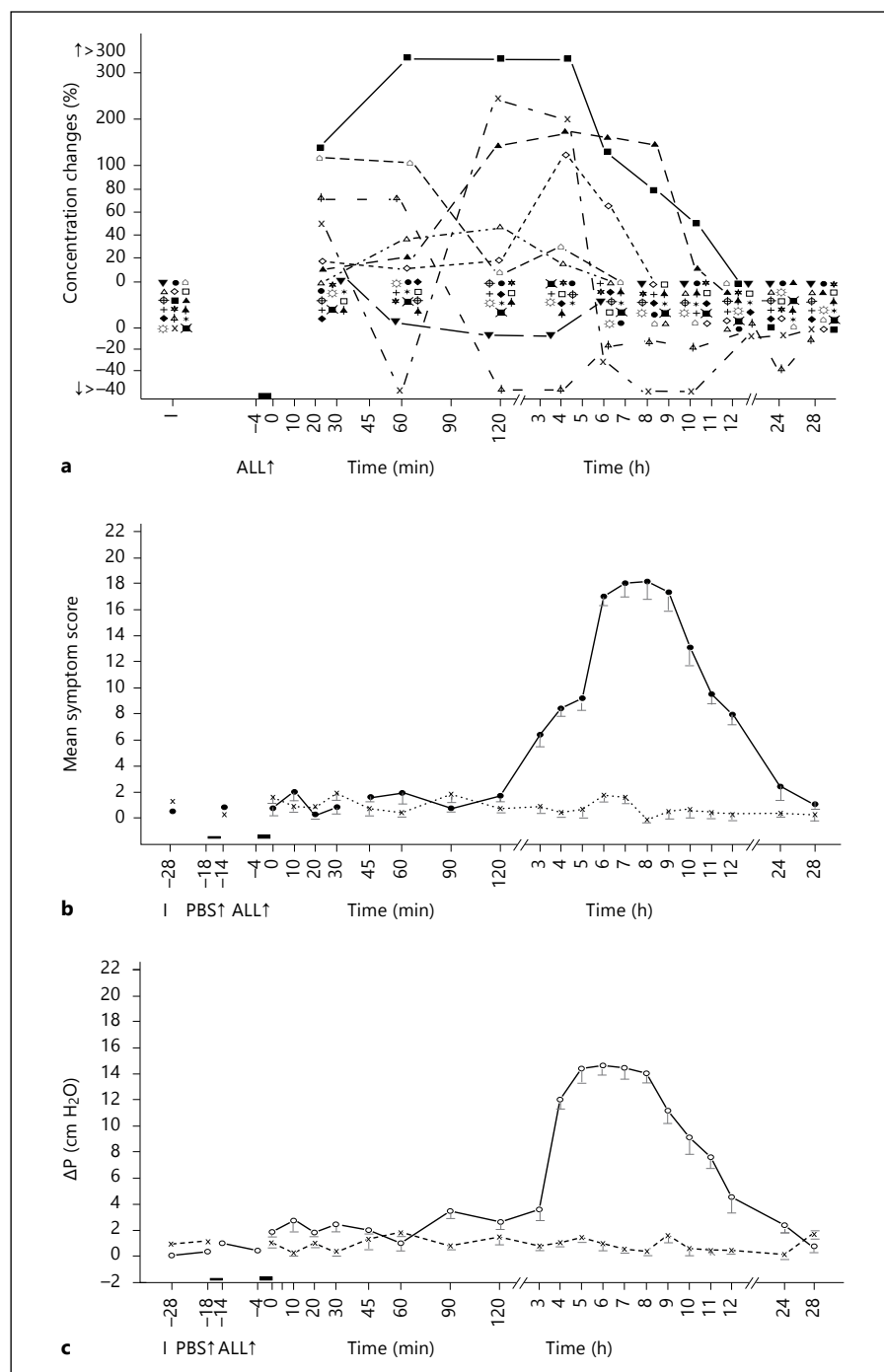


cosa, into the conjunctivae; (b) the local blood vessel, lymphatic and neurogenic network, facilitating the communication between both organs [2, 5–7, 18] (see further the online suppl. material). The primary allergic reactions in the nasal mucosa due to the intranasal exposure to an inhalant allergen can affect the conjunctivae in various

manners involving a number of mechanisms and factors, which are discussed in the online supplementary file.

Different hypersensitivity mechanisms activated by direct exposure of the conjunctiva to external allergens result then in the development of different types of primary KCR, such as an IKCR, LKCR or DYKCR [1–4,

Fig. 2. The SLKCRs (n = 16). I = Initial (baseline) values; ALL = allergen. **a** The mean score of particular factors during the SLKCR: Φ = IL-1 β ; $\square$ = IL-2; Δ = IL-3; $\blacksquare$ = IL-4; $\blacktriangle$ = IL-5; $\blacktriangleup$ = IL-6; $\star$ = IL-7; $\blacktriangleleft$ = IL-8; $\diamond$ = IL-10; $\blacksquare$ = IL-12p40; $\star$ = IL-12p70; $\odot$ = IL-13; $+$ = IL-18; $\blacklozenge$ = IFN- γ ; $\triangleleft$ = GM-CSF; $\times$ = G-CSF; $\blacktriangledown$ = TNF- α ; $\bullet$ = TGF- β . **b** The total mean score of keratoconjunctival signs and symptoms during the SLKCR ($\bullet$) and PBS control (x). **c** The mean rhinomanometric values (nasopharynx-nostril pressure gradients) recorded during the late nasal response ($\circ$) and PBS control (x).



8–17, 38, 41–43]. Similarly, different hypersensitivity mechanisms taking place in the nasal mucosa leading to the development of different types of nasal response can then, under certain circumstances, induce different types of the SKCR, such as an SIKCR, SLKCR or SDYKCR [7–12, 38]. The different types of the primary KCR as well as

of the SKCR are associated with different cytological profiles and with different profiles of various factors, such as mediators, cytokines, chemokines and adhesion molecules in the tears [4–7, 14]. The appearance of a cytokine in the tears of the patients suffering from the primary form of allergic keratoconjunctivitis has already been in-

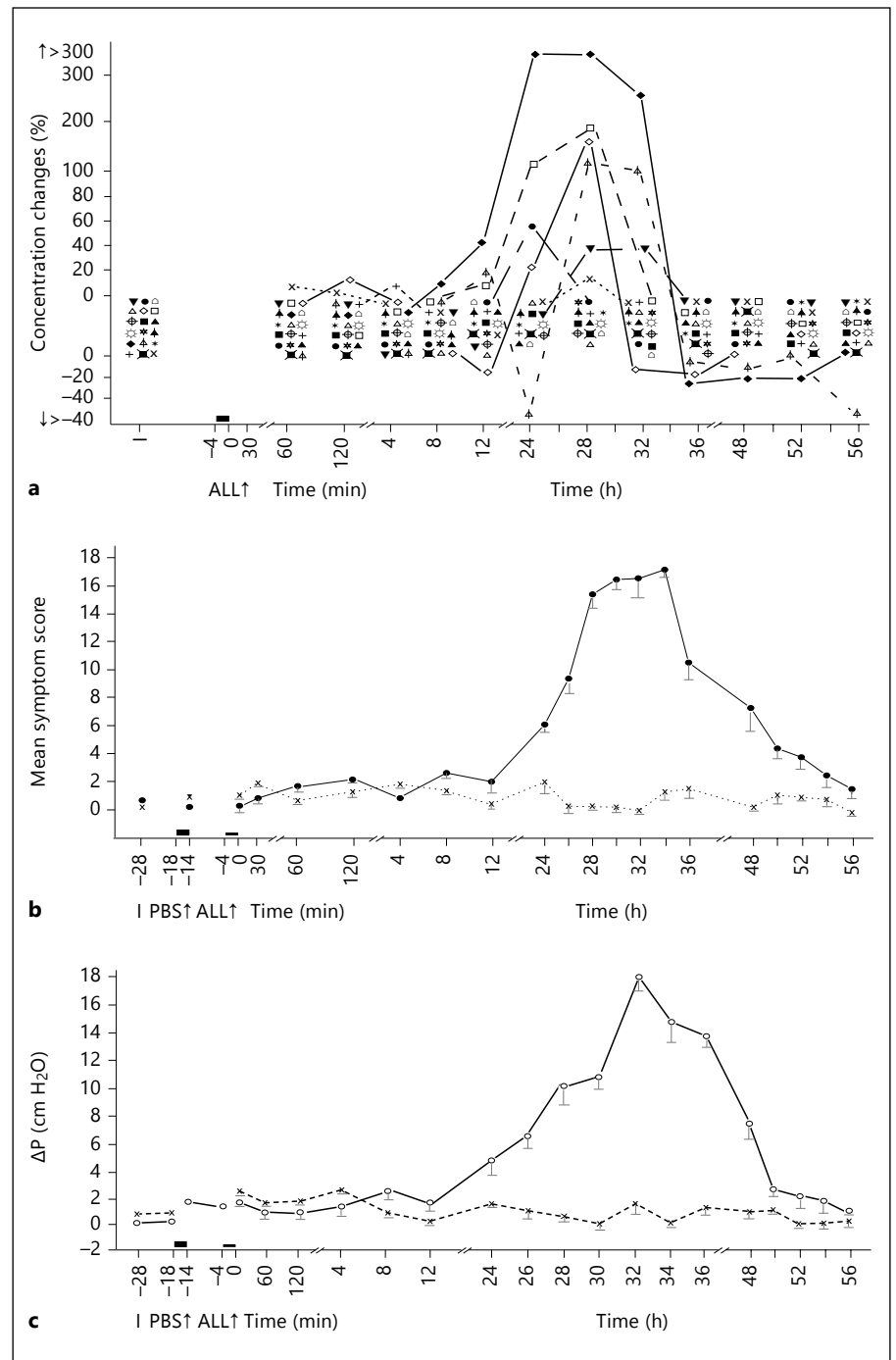


Fig. 3. The SDYKCRs (n = 12). I = Initial (baseline) values; ALL = allergen. **a** The mean score of particular factors during the SDYKCR: ϕ = IL-1 β ; $\square$ = IL-2; Δ = IL-3; $\blacksquare$ = IL-4; $\blacktriangle$ = IL-5; $\blacktriangleup$ = IL-6; $\star$ = IL-7; $\blacktriangleleft$ = IL-8; $\diamond$ = IL-10; $\blacksquare$ = IL-12p40; $\star$ = IL-12p70; $\odot$ = IL-13; $+$ = IL-18; $\blacklozenge$ = IFN- γ ; $\triangleleft$ = GM-CSF; $\times$ = G-CSF; $\blacktriangledown$ = TNF- α ; $\bullet$ = TGF- β . **b** The total mean score of keratoconjunctival signs and symptoms during the SDYKCR ($\bullet$) and PBS control ($\times$). **c** The mean rhinomanometric values (nasopharynx-nostril pressure gradients) recorded during the delayed nasal response ($\circ$) and PBS control ($\times$).

vestigated [9–13, 20, 37–41, 43]. In most of these studies the cytokines, such as IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IFN- γ and TNF- α , have been detected by means of a single, patient-related determination in the tears [3, 9–13, 20, 37–43]. No data concerning the concentration changes of cytokines in tears during the

particular types of the primary KCR to the conjunctival provocation tests with allergens, thus response-related data, are available in the literature. Moreover, little data is available concerning the possible occurrence of particular cytokines in the tears of healthy subjects, which could be exploited as reference parameters [20, 22, 23].

Table 2. Concentration (pg/ml) of particular cytokines in tears during the SIKCR (n = 15)

	Before the challenge	After the challenge							
		0–10 min	20–30 min	45 min	60 min	90 min	120 min	180 min	240 min
IL-4									
SICR	<0.6	2.1±0.9*	4.7±1.1*	4.5±0.4*	3.5±0.7*	1.5±0.6	<0.6	0.8±0.1	<0.6
PBS	<0.6	1.1±0.2	0.8±0.2	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
IL-6									
SICR	<4.0	<4.0	<4.0	5.9±0.5*	5.7±0.4*	4.3±0.2	<4.0	<4.0	<4.0
PBS	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0	<4.0
IL-10									
SICR	<3.0	4.2±0.7	7.3±1.4*	7.5±0.9*	3.8±0.6	<3.0	<3.0	<3.0	<3.0
PBS	<3.0	<3.0	<3.0	<3.0	3.3±0.2	<3.0	3.0±0.0	<3.0	<3.0
IL-12p70									
SICR	2.3±0.2	4.3±0.5*	2.5±0.2	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1
PBS	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1
GM-CSF									
SICR	3.5±0.4	4.4±1.0	5.9±1.0*	5.7±0.6*	6.4±1.5*	<3.0	<3.0	3.1±0.1	3.1±0.1
PBS	3.4±0.2	3.4±0.4	3.7±0.5	3.0±0.0	3.2±0.1	3.3±0.2	3.3±0.2	3.1±0.1	3.3±0.3

SICR = Secondary immediate conjunctival response; for other abbreviations, see table 1. Values are means ± SEM; * p < 0.05: statistical significance with respect to the baseline (before the challenge).

Table 3. Concentration (pg/ml) of particular cytokines in tears during the SLKCR (n = 16)

	Before the challenge	After the challenge									
		0.5 h	1 h	2 h	4 h	6 h	8 h	10 h	12 h	24 h	28 h
IL-3											
SLCR	<31.2	<31.2	43.5±2.5*	45.0±1.4*	35.6±0.3	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2
PBS	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2	<31.2
IL-4											
SLCR	<0.6	2.2±0.5	4.6±0.7*	5.5±1.0*	4.9±0.8*	2.3±0.5	1.8±0.4	1.5±0.5	<0.6	<0.6	<0.6
PBS	<0.6	1.0±0.3	1.0±0.3	0.8±0.1	1.0±0.4	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
IL-5											
SLCR	3.1±0.1	3.5±0.2	3.9±0.4	7.3±0.9*	8.5±1.0*	7.7±0.9*	7.2±0.6*	3.5±0.4	<3.0	<3.0	3.3±0.2
PBS	<3.0	<3.0	<3.0	3.1±0.1	3.1±0.1	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
IL-8											
SLCR	2.6±0.5	4.5±0.8*	4.5±0.6*	<1.3*	<1.3*	2.0±0.5	2.1±0.3	2.0±0.5	2.5±1.1	1.7±0.4	2.4±1.0
PBS	2.3±0.4	2.5±0.7	2.9±0.5	2.0±0.4	2.1±0.5	1.9±0.6	2.4±0.79	1.9±0.3	1.9±0.2	2.2±0.7	2.2±0.5
IL-10											
SLCR	3.1±0.1	3.7±0.5	3.6±0.5	3.9±0.8	7.2±1.4*	5.1±0.7*	3.3±0.2	<3.0	<3.0	<3.0	<3.0
PBS	<3.0	<3.0	<3.0	<3.0	3.3±0.1	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
GM-CSF											
SLCR	<3.0	6.5±0.9*	6.1±1.0*	3.2±0.1	3.9±1.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
PBS	<3.0	3.2±0.1	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
G-CSF											
SLCR	1.6±0.3	2.5±0.9	<0.8*	5.5±1.0*	4.6±0.5*	1.9±0.3	<0.8*	<0.8*	1.7±0.4	1.5±0.5	1.6±0.2
PBS	1.1±0.2	<0.8	0.9±0.1	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8
TNF-α											
SLCR	6.9±0.5	8.2±1.0*	7.3±0.9	<6.0*	<6.0*	6.3±0.1	6.7±0.4	6.5±0.2	6.4±0.4	6.8±0.6	6.7±0.3
PBS	6.3±0.2	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0

SLCR = Secondary late conjunctival response. Values are means ± SEM; * p < 0.05: statistical significance with respect to the baseline (before the challenge).

Table 4. Concentration (pg/ml) of particular cytokines in tears during the SDYKCR (n = 12)

		Before the challenge	After the challenge										
			12 h	24 h	26 h	28 h	30 h	32 h	34 h	36 h	48 h	52 h	56 h
IL-2													
	SDYCR	3.1±0.1	3.3±0.2	6.9±1.5*	7.5±0.8*	8.7±1.0*	8.3±0.5*	6.5±0.9*	<3.0	<3.0	<3.0	<3.0	<3.0
	PBS	<3.0	<3.0	3.1±0.1	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
IL-8													
	SDYCR	2.8±0.5	3.1±1.0	<1.3*	<1.3*	6.3±0.5*	5.9±0.8*	5.6±1.0*	2.6±0.5	2.5±0.4	2.4±0.3	2.5±0.6	<1.3*
	PBS	2.5±0.2	2.4±0.6	2.1±0.2	1.7±0.5	2.2±0.5	2.3±0.5	2.2±0.8	2.6±0.7	1.9±0.3	1.9±0.2	2.1±0.4	2.1±0.5
IL-10													
	SDYCR	3.7±0.2	3.0±0.3	4.5±1.0	8.2±0.5*	9.5±1.0*	9.4±0.7*	3.1±0.1	3.0±0.0	3.5±0.5	3.8±0.6	3.7±0.2	3.7±0.6
	PBS	3.4±0.3	<3.0	<3.0	<3.0	3.5±0.4	3.0±0.0	<3.0	<3.0	3.0±0.0	3.6±0.1	3.2±0.1	3.5±0.3
IFN-γ													
	SDYCR	1.4±0.2	2.0±1.0	7.0±0.9*	7.4±1.1*	6.6±1.3*	6.5±2.0*	4.9±0.7*	<1.0	<1.0	1.2±0.2	1.2±0.1	1.3±0.2
	PBS	1.1±0.1	1.3±0.2	1.5±0.4	1.5±0.3	1.0±0.0	2.0±0.3	1.4±0.3	1.6±0.5	1.3±0.2	1.5±0.3	1.4±0.2	1.5±0.3
TNF-α													
	SDYCR	<6.0	<6.0	<6.0	<6.0	8.5±0.7*	9.1±1.2*	8.6±0.9*	6.0±0.0	<6.0	<6.0	<6.0	<6.0
	PBS	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0
TGF-β													
	SDYCR	<6.0	<6.0	9.5±0.5*	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0
	PBS	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0	<6.0

SDYCR = Secondary delayed conjunctival response. Values are means ± SEM; * $p < 0.05$; statistical significance with respect to the baseline (before the challenge).

Unfortunately, the appearance of cytokines in tears during the particular types of the SKCR to NPTs has not yet been studied. The response-related results shown in this study are probably the first data on this topic to be reported in the literature. According to our results, the following hypothesis can be presumed: (a) in the SIKCR having been associated with significant concentration changes ($p < 0.05$) in tears of IL-4, IL-6, IL-10, IL-12p70 and GM-CSF, the involvement of the IgE-mediated hypersensitivity mechanism(s) upon participation of mast cells, eosinophils and Th2 lymphocytes can be expected; (b) in the SLKCR accompanied by significant concentration changes ($p < 0.05$) in tears of IL-3, IL-4, IL-5, IL-8, IL-10, GM-CSF, G-CSF and TNF-α, involvement of basophils, eosinophils, neutrophils and Th2 lymphocytes can be presumed; (c) in SDYKCR, associated with significant concentration changes ($p < 0.05$) in tears of IL-2, IL-8, IL-10, IFN-γ, TGF-β and TNF-α, an involvement of the cell-mediated hypersensitivity mechanism(s) upon participation of neutrophils, monocytes and Th1 lymphocytes, and probably also epithelial and endothelial cells, can be suspected.

The increased concentrations of some cytokines in tears may suggest their increased release from the activated particular cell types. In contrast, the decreased con-

centrations of other cytokines in tears, sometimes even under the detection limits, during certain stages of the particular SKCR types, would suggest either a recovery phase of the cells, after they had released their whole intracellular cytokine potential, or a temporary exhaustion of the cytokine-producing cells or a temporary blocking of the specific cytokines by their antagonist(s) [4–7, 14].

The low prechallenge cytokine concentrations in the tears would suggest their initial release in the nasal mucosa due to the primary allergic reaction in the nasal mucosa. This presumption may be supported by our previous data, showing only very limited numbers, even non-activated cells in the tears during the SKCR types [4–7].

Another interesting aspect concerns the comparison of the cytokine profiles recorded during SKCRs in this study with the cytokine profiles measured during the secondary conjunctival responses in the patients with allergic conjunctivitis, according to our preliminary, not yet published, data. Compared with corresponding secondary conjunctival responsetypes, the SKCRs demonstrated the following differences in the appearance and concentration changes of individual cytokines in tears: (a) in SIKCR, the absence of IL-3 but the additional presence of IL-6 and IL-12p70; (b) in SLKCR, the absence of IL-12p40 but the additional presence of TNF-α and G-CSF; (c) in

SDYKCR, the absence of G-CSF but the additional presence of TGF- β .

The origin of cytokines in the tears is another important aspect. They can be released by the genuine immunological mechanism(s) or by the reflex mechanisms. It is almost impossible to distinguish exactly which cytokine portion is caused by which mechanism.

However, applying a careful and nonirritant technique of tear collection, we are convinced that the cytokine concentrations measured in tears in the current study are certainly released by the immunological mechanism(s) taking place in the nasal mucosa. Moreover, even if some negligible reflex mechanism, in spite of the very gentle tear collection, has occurred, it would be of the same range throughout the whole study, thus without any further significant influence on the study results. On the other hand, no conjunctival biopsies were taken during the particular SKCR types, and no cytokine measurement in the nasal secretions simultaneously to their recording in tears was performed for technical reasons. These facts may be considered as a deficit of this study, and they may be included in the following research.

The conjunctival provocation tests with allergens, performed directly on the conjunctiva, confirm the role of an allergic reaction occurring in the conjunctiva due to a di-

rect exposure of conjunctival tissue to an inhalant allergen [3, 8, 12, 15–17, 21, 38, 42].

The conjunctival provocation tests are therefore suitable for demonstrating the primary types of KCR [3, 17, 41, 42]. On the other hand, the particular types of the SKCR, induced by an allergic reaction in the nasal mucosa, can only be confirmed by NPTs with allergens combined with simultaneous registration of the ocular features [4–7, 14].

However, a necessary condition for both kinds of provocation tests is the recording of the representative parameters before and repeatedly after the allergen challenge and that for a sufficiently long period of time, allowing the registration of the dynamic course of the entire particular response type. The provocation tests can then be combined with measurements of various supplementary parameters both in the tears and in the ocular tissues. Nevertheless, further research will be needed to clarify exactly the immunological mechanism(s) underlying the primary KCR as well as the SKCR types.

Disclosure Statement

The author has no conflict of interests to be disclosed.

References

- Bielory L, Friedlaender MH: Allergic conjunctivitis. *Immunol Allergy Clin North Am* 2008;28:43–57.
- Barney NP, Graziano FM, Cook EB, et al: 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 and Practice*, ed 7. Philadelphia, Mosby-Elsevier, 2009, pp 1117–1137.
- 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.
- Pelikan Z: *The Late Nasal Response*; thesis, Free University of Amsterdam, 1996.
- Pelikan Z: Seasonal and perennial allergic conjunctivitis: the possible role of nasal allergy. *Clin Exp Ophthalmol* 2009;37:448–457.
- Pelikan Z: Allergic conjunctivitis and nasal allergy. *Curr Allergy Asthma Rep* 2010;10:295–302.
- Pelikan Z: Cytologic changes in tears during the secondary conjunctival response induced by nasal allergy. *Br J Ophthalmol* 2012;96:941–948.
- Anderson DF: The conjunctival late-phase reaction and allergen provocation in the eye. *Clin Exp Allergy* 1996;26:1105–1107.
- Calder VL: Cellular mechanisms of chronic cell-mediated allergic conjunctivitis. *Clin Exp Allergy* 2002;32:814–817.
- Leonardi A, Fregona IA, Plebani M, et al: Th1- and Th2-type cytokines in chronic ocular allergy. *Graefes Arch Clin Exp Ophthalmol* 2006;244:1240–1245.
- Cook EB: Tear cytokines in acute and chronic ocular allergic inflammation. *Curr Opin Allergy Clin Immunol* 2004;4:441–445.
- Bacon AS, Ahluwalia P, Irani AM, et al: Tear and conjunctival changes during the allergen-induced early- and late-phase responses. *J Allergy Clin Immunol* 2000;106:948–954.
- Metz DP, Hingorani M, Calder VL, et al: T-cell cytokines in chronic allergic eye disease. *J Allergy Clin Immunol* 1997;100:817–824.
- Pelikan Z: The possible involvement of nasal allergy in allergic keratoconjunctivitis. *Eye* 2009;23:1653–1660.
- Friedlaender MH: Objective measurement of allergic reactions in the eye. *Curr Opin Allergy Clin Immunol* 2004;4:447–453.
- Shii D, Nakagawa S, Yoshimi M, et al: Inhibitory effects of cyclosporine A eye drops on symptoms in late phase and delayed-type reactions in allergic conjunctivitis models. *Biol Pharm Bull* 2010;33:1314–1318.
- Leonardi A: In-vivo diagnostic measurements of ocular inflammation. *Curr Opin Allergy Clin Immunol* 2005;5:464–472.
- Dua HS, Gomes JA, Donoso LA, Laibson PR: The ocular surface as part of the mucosal immune system: conjunctival mucosa-specific lymphocytes in ocular surface pathology. *Eye* 1995;9:261–267.
- Lambiase A, Normando EM, Vitiello L, et al: Natural killer cells in vernal keratoconjunctivitis. *Mol Vis* 2007;13:777–784.
- Leonardi A, Sathe S, Bartolotti M, et al: Cytokines, matrix metalloproteases, angiogenic and growth factors in tears of normal subjects and vernal keratoconjunctivitis patients. *Allergy* 2009;64:710–717.
- Melillo G, Bonini S, Cocco G, et al: Provocation tests with allergens. *Allergy* 1997;52(suppl 35):5–36.
- Carreño E, Enriquez-de Salamanca A, Teson M, et al: Cytokine and chemokine levels in tears from healthy subjects. *Acta Ophthalmol* 2010;88:250–258.
- Nakamura Y, Sotozono C, Kinoshita S: Inflammatory cytokines in normal human tears. *Curr Eye Res* 1998;17:673–676.

- 24 Pelikan Z: Mediators and some cytokines in tears during the late conjunctival response induced by primary allergic reaction in the nasal mucosa; in Pelikan Z (ed): *Conjunctivitis – A Complex and Multifaceted Disorder*. Rijeka, Intech, 2011, pp 57–74.
- 25 Pelikan M, Pelikan Z: The role of the nasal mucosa in some cases of allergic conjunctivitis and the effects of disodium cromoglycate (DSCG). *J Allergy Clin Immunol* 1985; 75(suppl to No 1):186.
- 26 Pelikan Z: Provocation tests (PT). I. Definition, reason, principles, indications, contraindications, conditions, requirements, contribution to the diagnostic procedures and therapeutic management. *Allergy (Cz)* 2007;9: 32–38.
- 27 Pelikan Z: The causal role of the nasal allergy in some patients with allergic conjunctivitis (abstract 739). *Allergy* 2002;57(suppl 73):230.
- 28 Pelikan Z: The role of allergy in sinus disease. Children and adults. *Clin Rev Allergy Immunol* 1998;16:55–156.
- 29 Houd DJ, Taylor CC: *Multivariate Analysis of Variance and Repeated Measures*. London, Chapman & Hall, 1987.
- 30 Sirigu P, Maxia C, Puxeddu R, et al: The presence of a local immune system in the upper blind and lower part of the human nasolacrimal duct. *Arch Histol Cytol* 2000;63:431–439.
- 31 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*, ed 3. Burlington, Elsevier Academic Press, 2005, pp 1477–1496.
- 32 Wotherspoon AC, Hardman-Lea S, Isaacson PG: Mucosa-associated lymphoid tissue (MALT) in the human conjunctiva. *J Pathol* 1994;174:33–37.
- 33 Youngman KR, Lazarus NH, Butcher EC: Lymphocyte homing: chemokines and adhesion molecules in T cell and IgA plasma cell localization in the mucosal immune system; in Mestecky J, Bienenstock J, Lamm M, Strober W, McGhee J, Mayer L (eds): *Mucosal Immunology*, ed 3. Burlington, Elsevier Academic Press, 2005, pp 667–680.
- 34 Hingorani M, Calder VL, Buckley RJ, et al: The role of conjunctival epithelial cells in chronic ocular allergic disease. *Exp Eye Res* 1998;67:491–500.
- 35 Leonardi A, Motterle L, Bortolotti M: Allergy and the eye. *Clin Exp Immunol* 2008; 153(suppl 1):17–21.
- 36 Choi SH, Bielory L: Late-phase reaction in ocular allergy. *Curr Opin Allergy Clin Immunol* 2008;8:438–444.
- 37 Leonardi A, Curnow SJ, Zhan H, et al: 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.
- 38 Vesaluoma M, Rosenberg ME, Teppo A, et al: Tumor necrosis factor alpha (TNF alpha) in tears of atopic patients after conjunctival allergen challenge. *Clin Exp Allergy* 1999;29: 537–542.
- 39 Uchino E, Sonoda S, Nakao K, et al: Alteration of tear cytokine balance by eye closure: analysis by multicytokine assay. *Graefes Arch Clin Exp Ophthalmol* 2006;244:747–749.
- 40 Sonoda S, Uchino E, Nakao K, et al: Inflammatory cytokine of basal and reflex tears analyzed by multicytokine assay. *Br J Ophthalmol* 2006;90:120–122.
- 41 Leonardi A: Vernal keratoconjunctivitis: pathogenesis and treatment. *Progr Retinal Eye Res* 2002;21:319–339.
- 42 Nivenius E, Van der Ploeg I, Gafvelin G, et al: Conjunctival provocation with airborne allergen in patients with atopic keratoconjunctivitis. *Clin Exp Allergy* 2012;42:58–65.
- 43 Metz DP, Bacon AS, Holgate ST, et al: Phenotypic characterization of T cells infiltrating the conjunctiva in chronic allergic eye disease. *J Allergy Clin Immunol* 1996;98:686–696.

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ON-LINE SUPPLEMENTS

CYTOKINES IN TEARS DURING THE SECONDARY KERATOCONJUNCTIVAL RESPONSES INDUCED BY ALLERGIC REACTION IN THE NASAL MUCOSA

METHODS

Patients

These patients underwent a routine diagnostic procedure consisting of : a detailed disease history, physical examination, basic laboratory and bacteriological tests, X-ray of paranasal sinuses, nasoscopy, skin tests with inhalant allergens, determination of serum immunoglobulins (Table 1), and ophthalmologic examination including ophthalmoscopy, slit-lamp evaluation, vital with fluorescein and cytologic screening of the tears.

Allergens

Dialyzed and lyophilized allergen extracts (Allergopharma, Reinbek, Germany) were diluted in phosphate-buffered saline (PBS) and used for skin tests in concentrations of 100-500 BU/mL and for NPTs in concentrations of 200-600 BU/mL (1:5 dilution) and 1000-3000 BU/mL (1:1= our standard concentrations) (Table 1S- On-line supplement), as recommended by the manufacturer.

Skin tests

Skin prick tests (SPT) with allergenic extracts in concentrations of 500 BU/mL were evaluated after 20 minutes. The intracutaneous tests in concentrations of 100-300 BU/mL were evaluated 20 minutes and 6, 12, 24, 36, 48, 56, 72 and 96 hours after the intradermal injection. A skin flare and wheal (>7.0 mm in diameter) occurring after 20 minutes was considered to be a positive immediate skin response, the skin infiltration appearing between 6 and 12 hours to be a late skin response, and the skin induration recorded later than 48 hours to be a delayed skin response [4-7, 14, 31-35].

Nasal provocation tests (NPTs)

NPTs with allergens were performed by means of rhinomanometry, described in our previous studies [4-7, 14, 28, 31-35]. The nasal obstruction due to the nasal mucosa edema was evaluated by means of nasopharynx-nostril pressure gradients (NPG), being the pressure differences (ΔP) between the nasopharyngeal cavity and the outside air, expressed in cm H₂O. NPTs were performed according the following schedule: (1) baseline values recorded at 0, 5 and 10 minutes; (2) PBS control values recorded at 0, 5 and 10 minutes after a 3-minute application of PBS to the nasal mucosa of the non-intubated nasal cavity by means of a saturated wad of cotton wool on a nasal probe inserted under the middle turbinate; (3) post-challenge values recorded after a 3-minute challenge with allergen, performed in the same manner as the challenge with PBS, at 0, 5, 10, 20, 30, 45, 60, 90 and 120 minutes, then every hour up to the 12th hour, and every second hour during the time-periods between the 24th-38th and 48th-56th (60th) hour [4-7, 14, 28, 31-35].

The allergens used for the NPTs were chosen with respect to the disease history and/or skin tests (Tables 1, 1S). All NPTs were performed with allergenic extracts firstly in 1:5 dilution and if they were negative, then with extracts in 1:1 dilution (our basic concentrations) (Table 1S- On-line supplement).

The nasal response was assessed to be positive when the mean post-challenge NPG values increased by at least 2.0 cm H₂O (1.2 ± 0.3 , mean $\pm$ SE) with respect to the baseline values, recorded at least at three consecutive time intervals [7-12, 3-35]. The NPG changes recorded within 60-120 minutes after the allergen challenge were considered to be an immediate (INR), those recorded within 4-12 hours to be a late (LNR), and the changes measured later than 24 hours to be a delayed (DYNR) response [7-12, 3-35].

Control challenge with phosphate-buffered saline (PBS)

The control nasal challenges with PBS were performed by the same schedule as that used for the NPTs with allergens.

Detection limits of cytokine immunoassay kits

The detection limits cytokine immunoassay kits in *pg/mL* are reported in parentheses. The cytokines IL-1 β (1.0), IL-2 (<3.0), IL-3 (31.2), IL-5 (3.0), IL-6 (4.0), IL-7 (0.1), IL-10 (<3.0), IL-13 (<3.0), GM-CSF (<3.0), G-CSF (0.8), TNF- α (6.0), TGF- β (6.0) were measured by means of R & D System (Minneapolis/MN, USA) kits, IL-4 (0.6), IL-8 (1.3), IL-12p70 (2.1), IL-18 (9.2) and IFN- γ (1.0) by Bender MedSystems (Wien, Austria), and IL-12p40 (3.9) by Becton Dickinson (San Jose/CA, USA) kits. The inter-assay as well as intra-assay coefficients of variations for these kits were less than 10%.

Additional statistical analysis

The initial and repeated nasal and the ocular responses (mean total symptom scores) and the PBS control challenges in individual patients were statistically evaluated by the generalized multivariate analysis of variance model (MANOVA) [36]. The polynomials were fitted to the mean curves over time (8 time points within 120 minutes and 25 time points up to 56 hours after the allergen challenge) and the hypotheses were tested by the modified MANOVA computerized system [36].

RESULTS

Nasal responses (NR)

In 43 patients 101 NPTs with various inhalant allergens (Table 1S) and 43 PBS control challenges were performed. They developed 43 positive nasal responses, 15 immediate (INR; $p < 0.001$), 16 late (LNR; $p < 0.001$), 12 delayed (DYNR; $p < 0.05$) and 58 negative (NNR) nasal responses ($p > 0.1$) (Table 1S). All 43 PBS control tests were negative ($p > 0.2$). The particular NR types did not differ with respect to the individual allergens ($p > 0.2$). The repeated NPTs resulted in the development of the similar NR types (Figures 1C, 2C, 3C). No statistically significant differences were found between the initial and the repeated responses ($p > 0.2$).

DISCUSSION

Relationship conjunctivae and nose

The primary allergic reactions in the nasal mucosa due to the intranasal exposure to an external inhalant allergen can affect the conjunctivae in various manners involving a number of mechanisms and factors, such as (1) various mediators, cytokines and cellular constituents, released during the allergic reaction in the nasal mucosa, can reach the conjunctiva either indirectly having been transported by the local blood vessels, such as *arteriae maxillaris- pars pterygo- palatina*, *venae facialis*, *plexus pterygoideus*, or under certain conditions directly by the retrograde penetration through the naso-lacrimal duct; (2) the blood cells, participating

directly in the allergic reactions or the cells having been released from the nasal mucosa during the allergic reaction, can reach the conjunctival tissue either through the blood and/or lymphatic system or being transported by the nasal secretions or under some circumstances, they can migrate through the naso-lacrimal duct into the conjunctivae [4-7,31-34]; (3) the allergic reactions taking place in the nasal mucosa can also stimulate the local neurogenic network, such as *nervus trigemini*, *nervus nasociliaris*, *ganglion pterygo-palatinum*, releasing thereafter various neuropeptides reaching conjunctiva along and/or through the particular nerves; (4) the factors released by the allergic reaction in the nasal mucosa can also affect and stimulate the nose- associated lymphatic tissue (NALT), being linked to the other eye-related lymphatic tissues, such as the tear duct-associated lymphatic tissue (TALT), lacrimal drainage-associated lymphatic tissue (LDALT), conjunctiva- associated lymphatic tissue (CALT) and eye-associated lymphatic tissue (EALT) [4-7,18,31,32,34,37-40]. These abundant lymphatic tissue network facilitates not only transmission of various membrane-bound receptor signals but also cellular traffic among the related as well as adjacent tissue types [4-7, 18, 31-34, 37-40]; (5) the so-called “defective homing mechanism” of lymphocytes can also be sometimes involved in the communication between the conjunctival tissue and nasal mucosa. Cell traffic of the B- and T- lymphocytes is maintained and regulated not only by attraction mechanisms, governed by chemotactic factors, cytokines, lymphokines and adhesion molecules, but also through the so-called “homing” mechanism, controlled by a number of homing factors. The particular sub-sets of lymphocytes activated initially in one tissue, after migrating into the blood stream and/or lymphatic network to finish their maturation, do not return to this original tissue, but due to the disturbed homing factors they migrate into other tissues in different localities [4-6,40]; (6) various additional factors and mechanisms could, under certain circumstances, influence the relation of nasal mucosa and conjunctiva, such as increased intranasal pressure caused by edema of the nasal mucosa, disturbed air passage through the nose and disturbance or partial stagnation of the tear drainage through the ductus naso-lacrimalis [3-7, 9, 10, 14, 23,31, 37,41]. In the primary as well as in the secondary (induced) form of the allergic keratoconjunctivitis various hypersensitivity mechanisms, such as immediate (IgE-mediated, Type I), late (Type III) or delayed (cell-mediated, Type IV), can be involved [1-25, 42-49]. The particular cell types as well as the above mentioned factors appearing in tears during a certain keratoconjunctival response type may be of different origin and can execute different functions. They can be either an integrated part of the allergic reaction and after fulfilling their specific role the cells migrate actively or are expelled as a superfluous component into the tears acting here as a waste and drainage medium. The factors are released directly into the tears, as a direct consequence of the positive allergic reaction [2-7, 14, 31].

REFERENCES

31. Pelikan Z. Mediators and some cytokines in tears during the late conjunctival response induced by primary allergic reaction in the nasal mucosa. In: Pelikan Z (Ed). *Conjunctivitis- a complex and multifaceted disorder*. Rijeka (Croatia): InTech 2011; 57-74
32. Pelikan M, Pelikan Z. The role of the nasal mucosa in some cases of allergic conjunctivitis and the effects of Disodium Cromoglycate (DSCG). *J Allergy Clin Immunol* 1985; 75 (Suppl to No 1): 186
- 33 Pelikan Z. Provocation tests (PT). I. Definition, reason, principles, indications, contra-indications, conditions, requirements, contribution to the diagnostic procedures and therapeutic management. *Allergy (Cz)*, 9 (No.1): 32- 38; 2007
- 34 Pelikan Z. The causal role of the nasal allergy in some patients with allergic conjunctivitis. *Allergy*, 57 (Suppl.73): 230: . (Abstr.739) ; 2002
- 35 Pelikan Z. The role of allergy in sinus disease. Children and adults. *Clin Rev Allergy Immunol*, 16: 55-156; 1998
36. Houd DJ, Taylor CC. *Multivariate analysis of variance and repeated measures*. London: Chapman & Hall; 1987
37. Sirigu P, Maxia C, Puxeddu R et al. The presence of a local immune system in the upper blind and lower part of the human nasolacrimal duct. *Arch Histol Cytol* 2000; 63: 431-439
38. 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 Ed. Burlington/ MA, San Diego /CA, London: Elsevier-Academic Press 2005; 1477-1496
39. Wotherspoon AC, Hardman-Lea S, Isaacson PG. Mucosa-associated lymphoid tissue (MALT) in the human conjunctiva. *J Pathol* 1994; 174: 33-37
40. Youngman KR, Lazarus NH, Butcher EC. Lymphocyte homing: chemokines and adhesion molecules in T cell and IgA plasma cell localization in the mucosal immune system. In: Mestecky J, Bienenstock J, Lamm M, Strober W, McGhee J, Mayer L (Eds). *Mucosal Immunology*, 3rd Ed. Burlington/MA, San Diego /CA, London: Elsevier Academic Press 2005; 667-680
41. Hingorani M, Calder VL, Buckley RJ, et al. The role of conjunctival epithelial cells in chronic ocular allergic disease. *Exp Eye Res* 1998; 67: 491-500
42. Leonardi A, Motterle L, Bortolotti M. Allergy and the eye. *Clin Exp Immunol* 2008; 153 (Suppl 1): 17-21
43. Choi SH, Bielory L. Late-phase reaction in ocular allergy. *Curr Opin Allergy Clin Immunol* 2008; 8: 438-444
44. Peltonen S, Kari O, Jarva H et al. Complement activation in tear fluid during occupational mould challenge. *Ocular Immunol Inflamm* 2008; 16: 224-229
45. Leonardi A, Bogacka E, Fauquert JL, et al. Ocular allergy: recognizing and diagnosing hypersensitivity disorders of the ocular surface. *Allergy* 2012; 67: 1327-1337
46. Uchio E, Ono SY, Ikezawa Z, et al. 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
47. Guglielmetti S, Dart JKG, Calder V. Atopic keratoconjunctivitis and atopic dermatitis. *Curr Opin Allergy Clin Immunol*

2010; 10: 478-485

48. Shoji J, Inada N, Sawa M. Antibody array-generated cytokine profiles of tears of patients with vernal keratoconjunctivitis or giant papillary conjunctivitis. *Jpn J Ophthalmol* 2006; 50: 195-204
49. 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 Ed. St.Louis (MI, USA): Mosby-Year Book, Inc 1993; 595-637.
50. Abelson M, Howes J, George M. The conjunctival provocation test model of ocular allergy: Utility for assessment of an ocular corticosteroid, Loteprednol etabonate. *J Ocular Pharmacol & Therap* 1998; 14: 533-542
51. Abelson MB, Chambers WA, Smith LM. Conjunctival allergen challenge. A clinical approach to studying allergic conjunctivitis. *Arch Ophthalmol* 1990; 108: 84-88

TABLE 1 S . Survey of the allergens used for the nasal challenges

Allergen	Concentration BU/mL	Nasal responses positive (n=43)	AKC (n=24)	VKC (n=19)	Keratoconjunctival responses			
					Positive (n=43)			Negative (n=58)
					SIKCR (n=15)	SLKCR (n=16)	SDYKCR (n=12)	
<i>Dermatophagoides pteron</i>	1000	4	4		1	2	1	3
<i>Dermatophagoides farinae</i>	1000	1	1		1	0	0	1
Animal danders								
- dog	3000	2	2		1	1	0	2
- cat	2000	3	3		1	1	1	5
- guinea pig	2000	1	1		0	1	0	3
- hamster	2000	1	1		1	0	0	2
- cow	2000	1	1		0	1	0	2
- horse	1000	1	1		0	0	1	2
- goat	1000	1	1		1	0	0	0
Feather								
- canary	3000	2	2		1	1	0	3
- parrot	3000	2	2		0	1	1	4
- parakeet	2000	1	1		0	0	1	1
- hen	2000	1	1		0	1	0	0
- duck	1000	1	1		0	0	1	1
<i>Aspergillus fumigatus</i>	1000	1	1		0	1	0	3
<i>Aspergillus niger</i>	1000	1	1		0	1	0	3
Pollen								
- grass mix I	1000	4		4	1	2	1	5
- grass mix II	1000	1		1	1	0	0	2
- flower mix	5000	1		1	0	0	1	2
- tree mix	3000	2		2	1	0	1	5
- weed mix	1000	1		1	1	0	0	0
- birch	1000	3		3	1	1	1	1
- willow	1000	1		1	0	1	0	2
- hazel	1000	2		2	1	0	1	2
- poplar	1000	1		1	0	1	0	2
- ragweed giant	1000	2		2	1	0	1	1
- ragweed short	1000	1		1	1	0	0	1

BU/mL = Biologic units per millilitre; SAC=Sesonal allergic conjunctivitis; PAC= Perennial allergic conjunctivitis;
 SICR=secondary immediate conjunctival response; SLCR= secondary late conjunctival response; SDYCR= secondary
 delayed conjunctival response

Grasspollen mix I= *Dactylis glomerata*, *Lolium perenne*, *Phleum pratensis*, *Poa pratensis*;

Grasspollen mix II=*Festuca pratensis*, *Holcus lanatus*, *Agrostis alba*, *Anthoxanthum odoratum*

Flower pollen mix=*Dahlia variabilis*, *Solidago virgaurea*, *Primula variabilis*, *Forsythia suspensa*

Tree pollen mix= *Betula pendula*, *Corylus avellana*, *Juniperus communis*, *Salix alba*

Weed pollen mix= *Artemisia vulgaris*, *Plantago lanceolata*, *Rumex acetosa*, *Taraxacum officinale*

Table 2 S. Survey of Abelson's conjunctivitis grading scale and our "modified" keratoconjunctivitis symptom score ("Pelikan's modified grading scale")

	Abelson ^I	Abelson ^{II}	Pelikan ^{III}
I. CONJUNCTIVA-related			
. Objective signs			
- Hyperemia (injection, redness)	0 - 4	0 - 3	0 - 4
- Chemosis		0 - 3	0 - 4
- Hyperlacrimation (tearing)		0 - 3	0 - 4
- Palpebral edema			0 - 4
- Cobblestoning			0 - 1
Subjective symptoms			
- Itching (burning)	0 - 4	0 - 4	0 - 4
- Photophobia			0 - 4
- Blurred vision			0 - 4
II. CORNEA-related			
Objective signs			
- Horner-Trantas dots			0 - 1
- Diminished sheen, transparency, reflexion			0 - 2
- Epithelial defects			0 - 2
- Scars			0 - 1
Subjective symptoms			
- Foreign body feeling			0 - 1
- Ocular pain			0 - 2

References: I= 2, 50; II= 51; III=5,6,14, 31

Abelson's grading scale: 0=None; 1=Mild (intermittent); 2=Moderate; 3=Severe; (4=extremely severe or "incapacitating" "itching"); [Significant threshold: ≥ 2]

Pelikan's grading scale:

- (I) Conjunctiva-related signs and symptoms : 0=Absent; 1=Mild (present to a slight degree or intermittent); 2=Moderate; 3= Pronounced (moderately severe); 4=Severe;
 (II) Cornea-related signs and symptoms: 0 = Absent; 1= Present/intermittent; 2= Pronounced/ continuously present

[Differences of ≥ 6 or more points (5 ± 1 , mean $\pm$ SE) with respect to the pre-challenge value, recorded at least at 3 consecutive time-intervals, were found to be statistically significant ($p < 0.05$)].⁴⁻⁷

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Figure 1 (A, B, C) is on a separate supplementary sheet

Figure 2 (A, B, C) is on a separate supplementary sheet

Figure 3 (A, B, C) is on a separate supplementary sheet

ON-LINE SUPPLEMENTS

CYTOKINES IN TEARS DURING THE SECONDARY KERATOCONJUNCTIVAL RESPONSES INDUCED BY ALLERGIC REACTION IN THE NASAL MUCOSA

TABLE 2 S . Survey of the allergens used for the nasal challenges

Allergen	Concentration BU/mL	Nasal responses positive (n=43)	AKC (n=24)	VKC (n=19)	Keratoconjunctival responses			Negative (n=58)
					Positive (n=43)			
					SIKCR (n=15)	SLKCR (n=16)	SDYKCR (n=12)	
<i>Dermatophagoides pteron</i>	1000	4	4		1	2	1	3
<i>Dermatophagoides farinae</i>	1000	1	1		1	0	0	1
.								
Animal danders								
- dog	3000	2	2		1	1	0	2
- cat	2000	3	3		1	1	1	5
- guinea pig	2000	1	1		0	1	0	3
- hamster	2000	1	1		1	0	0	2
- cow	2000	1	1		0	1	0	2
- horse	1000	1	1		0	0	1	2
- goat	1000	1	1		1	0	0	0
Feather								
- canary	3000	2	2		1	1	0	3
- parrot	3000	2	2		0	1	1	4
- parakeet	2000	1	1		0	0	1	1
- hen	2000	1	1		0	1	0	0
- duck	1000	1	1		0	0	1	1
<i>Aspergillus fumigatus</i>	1000	1	1		0	1	0	3
<i>Aspergillus niger</i>	1000	1	1		0	1	0	3
Pollen								
- grass mix I	1000	4		4	1	2	1	5
- grass mix II	1000	1		1	1	0	0	2
- flower mix	5000	1		1	0	0	1	2
- tree mix	3000	2		2	1	0	1	5
- weed mix	1000	1		1	1	0	0	0
- birch	1000	3		3	1	1	1	1
- willow	1000	1		1	0	1	0	2
- hazel	1000	2		2	1	0	1	2
- poplar	1000	1		1	0	1	0	2
- ragweed giant	1000	2		2	1	0	1	1
- ragweed short	1000	1		1	1	0	0	1

BU/mL = Biologic units per millilitre; SAC=Sesonal allergic conjunctivitis; PAC= Perennial allergic conjunctivitis;
 SICR=secondary immediate conjunctival response; SLCR= secondary late conjunctival response; SDYCR= secondary
 delayed conjunctival response

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Tree pollen mix= *Betula pendula*, *Corylus avellana*, *Juniperus communis*, *Salix alba*

Weed pollen mix= *Artemisia vulgaris*, *Plantago lanceolata*, *Rumex acetosa*, *Taraxacum officinale*

LEGEND

Figure 1. The secondary immediate keratoconjunctival responses (SIKCRs; n=15)

- A.** The mean score of particular factors during the SIKCR: $\oplus$ = IL-1 β , $\square$ = IL-2, Δ = IL-3, $\blacksquare$ = IL-4, $\blacktriangle$ = IL-5, $\blacklozenge$ = IL-6, $\ast$ = IL-7, $\clubsuit$ = IL-8, $\diamond$ = IL-10, $\boxtimes$ = IL-12p40, $\star$ = IL-12p70, $\circ$ = IL-13, $+$ = IL-18, $\blacklozenge$ = IFN- γ , $\triangleleft$ = GM-CSF, $\times$ = G-CSF, $\blacktriangledown$ = TNF- α , $\bullet$ = TGF- β
- B.** The total mean score of keratoconjunctival signs and symptoms during the SIKCR ($\bullet$) and PBS ($\times$)
- C.** The mean rhinomanometric values (NPG) recorded during immediate nasal response ($\circ$) and PBS ($\times$)
- I = Initial (baseline) values; PBS = Phosphate buffered saline; ALL = Allergen

Figure 2. The secondary late keratoconjunctival responses (SLKCRs; n=16)

- A.** The mean score of particular factors during the SLKCR: $\oplus$ = IL-1 β , $\square$ = IL-2, Δ = IL-3, $\blacksquare$ = IL-4, $\blacktriangle$ = IL-5, $\blacklozenge$ = IL-6, $\ast$ = IL-7, $\clubsuit$ = IL-8, $\diamond$ = IL-10, $\boxtimes$ = IL-12p40, $\star$ = IL-12p70, $\circ$ = IL-13, $+$ = IL-18, $\blacklozenge$ = IFN- γ , $\triangleleft$ = GM-CSF, $\times$ = G-CSF, $\blacktriangledown$ = TNF- α , $\bullet$ = TGF- β
- B.** The total mean score of keratoconjunctival signs and symptoms during the K ($\bullet$) and PBS ($\times$)
- C.** The mean rhinomanometric values (NPG) recorded during late nasal response ($\circ$) and PBS ($\times$)
- I = Initial (baseline) values; PBS = Phosphate buffered saline; ALL = Allergen

Figure 3. The secondary delayed keratoconjunctival responses (SDYKCRs; n=12)

- A.** The mean score of particular factors during the SDYKCR: $\oplus$ = IL-1 β , $\square$ = IL-2, Δ = IL-3, $\blacksquare$ = IL-4, $\blacktriangle$ = IL-5, $\blacklozenge$ = IL-6, $\ast$ = IL-7, $\clubsuit$ = IL-8, $\diamond$ = IL-10, $\boxtimes$ = IL-12p40, $\star$ = IL-12p70, $\circ$ = IL-13, $+$ = IL-18, $\blacklozenge$ = IFN- γ , $\triangleleft$ = GM-CSF, $\times$ = G-CSF, $\blacktriangledown$ = TNF- α , $\bullet$ = TGF- β
- B.** The total mean score of keratoconjunctival signs and symptoms during the SDYKCR ($\bullet$) and PBS ($\times$)
- C.** The mean rhinomanometric values (NPG) recorded during delayed nasal response ($\circ$) and PBS ($\times$)
- I = Initial (baseline) values; PBS = Phosphate buffered saline; ALL = Allergen