

ORIGINAL ARTICLE

Cytokine Profiles in Tears Accompanying the Secondary Conjunctival Responses Induced by Nasal Allergy*

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ABSTRACT

Purpose/aim: Allergic conjunctivitis (AC) occurs either in a primary form, due to the allergic reaction localized in the conjunctivae or in a secondary form, induced by an allergic reaction initiated primarily in the nasal mucosa. The purpose of this study was to investigate the cytokine profiles in tears associated with the secondary conjunctival response (SCR) types.

Materials and methods: In 47 AC patients developing 16 immediate (SICR; $p < 0.01$), 20 late (SLCR; $p < 0.001$) and 11 delayed (SDYCR; $p < 0.05$) responses to nasal provocation tests (NPTs) with allergens, the NPTs were repeated and combined with recording of cytokine concentrations in the tears.

Results: The SCRs were associated with significant concentration changes of particular cytokines in tears ($p < 0.05$) as follows: (1) SICRs: interleukin (IL)-3, IL-4, IL-10 and granulocyte macrophage colony-stimulating factor (GM-CSF); (2) SLCRs: IL-3, IL-4, IL-5, IL-8, IL-10, IL-12p40, GM-CSF and granulocyte colony-stimulating factor (G-CSF); and (3) SDYCRs: IL-2, IL-8, IL-10, interferon gamma, G-CSF and tumor necrosis factor alpha. No significant cytokine changes were recorded in tears during the phosphate-buffered saline controls or negative SCRs.

Conclusions: Different cytokine profiles in the tears accompanying the immediate, late and delayed types of SCR, induced by nasal allergy, would indicate involvement of different hypersensitivity mechanisms in the particular SCR types. The low cytokine concentrations in tears recorded during the SCRs may suggest their origin from the nasal mucosa. These results emphasize the diagnostic value of NPTs with allergens combined with monitoring of various ocular features in patients suffering from the secondary form of AC. These results may also have an impact on the therapeutical approach to this clinical entity.

Keywords: Allergic conjunctivitis, cytokines in tears, nasal allergy, nasal provocation tests with allergens, secondarily induced conjunctival responses

INTRODUCTION

Diseases of conjunctiva in which an allergic component plays a pivotal role include five clinical entities, divided into three groups as follows: (a) allergic conjunctivitis (AC): the seasonal allergic conjunctivitis (SAC) and the perennial allergic conjunctivitis (PAC) type, where only conjunctiva is involved, (b) allergic

keratoconjunctivitis: vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC), where besides the conjunctiva also cornea is affected, and (c) giant papillary conjunctivitis.^{1–6}

With respect to the location of the initial allergic reaction, two forms of AC can be distinguished: a primary AC form, where the initial allergic reaction takes a place in the conjunctiva due to the

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direct exposure of conjunctiva to an inhalant allergen, and a secondary AC form, where the allergic reaction occurring primarily in the nasal mucosa, due to an exposure of the nasal mucosa to an external allergen, induces a secondary conjunctival response (SCR).⁷⁻¹³

Various hypersensitivity types, such as immediate (IgE-mediated, type I), late (type III) or delayed (cell-mediated, type IV) can be involved in the mechanism(s) underlying both the AC forms. The participation of different hypersensitivity mechanisms, both in the primary and in the secondary AC form, results in development of different types of conjunctival response (CR), such as an immediate/early (ICR), late (LCR) or delayed (DYCR) response.^{1,3,5,7-21}

Despite abundant data in the literature concerning the appearance of cytokines in tears of AC patients, there is a dearth of information related to the dynamic aspects of the cytokine changes in tears during the individual CR types of the primary AC form.^{1-10,15-17,19-28} Moreover, no data at all are available to illustrate the appearance and concentration changes of the cytokines in tears accompanying the particular types of the SCR.⁹⁻¹¹

In our previous work, we have demonstrated different cytological features⁸⁻¹³ as well as different mediator patterns^{8,9,12} in the tears associated with the particular SCR types induced by nasal allergy.

The purpose of this study was (1) to investigate the changes in concentrations of particular cytokines during the individual SCR types, which means their dynamic course and (2) to evaluate the possible significance of the concentration changes of particular cytokines in tears for the possible hypersensitivity mechanisms underlying the individual secondary CR types.

METHODS

Patients

Forty-seven patients suffering from allergic conjunctivitis (AC) (AC=21 SAC and 26 PAC) for longer than three years and responding insufficiently to the topical ophthalmologic treatment, referred to our Department of Allergology & Immunology (Institute of Medical Sciences "De Klokkenberg", Breda, The Netherlands) for further analysis of their conjunctivitis complaints, volunteered to participate in this study.

These patients, 22 men and 25 women, 17-53 years of age (Table 1) had previously been treated with topical as well as oral H1 receptor antagonists, topical glucocorticosteroids vasoconstrictors and decongestant, however, without significant therapeutic effects. None of them had previously been treated

with immunotherapy. They all demonstrated normal intraocular pressure. In 11 of these patients, 14 previously performed conjunctival provocation tests (CPT) with inhalant allergens were negative. The routine diagnostic procedure, performed in these patients, consisted of the following: 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 and ophthalmologic examination including ophthalmoscopy, slit-lamp evaluation, vital staining with fluorescein and cytologic examination of the tears.

The routine diagnostic procedure performed in these 47 patients revealed positive or suspected history for nasal allergy (96%), positive skin tests with various inhalant allergens (100%), hyperaemic and edematous nasal mucosa (93%), conjunctival hyperaemia and tearing to a slight degree (100%), appearance of incidental eosinophils and conjunctival epithelial cells in the tears (73%), increased blood eosinophilia (18%) and positive specific IgE in the serum for some inhalant allergens (19%) (Table 1).

In these 47 patients, 98 nasal provocation tests (NPTs) with inhalant allergens (Table 2) and 47 control challenges with phosphate-buffered saline (PBS) performed by means rhinomanometry^{7-13,29,30} were combined 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 ($n=27$), topical cromolyn ($n=9$) and glucocorticosteroids ($n=11$) were withdrawn four weeks, whereas other treatments 48 h, before the study. The 47 positive NPTs producing a SCR of any type and 47 PBS controls were repeated 2-3 weeks later and supplemented with collection of the tears samples for the cytokine determination.

A 4-day interval was always inserted between the subsequent tests to prevent the carry-over effects and to allow for patient's recovery. The study protocol was approved by the local ethical committee, and an informed consent was obtained from all study participants. The study has been carried out according to the Declaration of Helsinki concerning the "Principles for medical research involving the human subjects".

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 200-600 BU/mL (1:5 dilution) and 1000-3000 BU/mL

TABLE 1 Characteristics of the patients.

	Patients (n = 47)			Control subjects n = 20
	SICR (n = 16)	SLCR (n = 20)	SDYCR (n = 11)	
Age (years)	26 ± 4	31 ± 8	29 ± 5	32 ± 3
Sex (M/F)	7/9	10/10	5/6	9/11
Disease history (years)	3.6 ± 1.1	3.9 ± 0.5	3.7 ± 1.0	0
Blood leukocyte count ($\times 10^9/L$) ^a	7.3 ± 0.8	8.2 ± 1.1	7.5 ± 1.3	7.8 ± 0.9
Blood eosinophil count ($\times 10^6/L$) ^b	322 ± 19	314 ± 23	288 ± 17	274 ± 31
Increased total IgE in the serum ^c	0	0	0	0
Positive specific IgE in the serum ^d	4	5	0	3
Positive skin response ^e				
Immediate type	9	8	1	6
Late type	7	11	3	10
Delayed type	0	1	7	4
Nasal histamine threshold ^f				
decreased	8	6	2	9
normal	8	14	9	11
Nasal provocation tests				
Positive	16	20	11	20
Negative	13	17	12	15
Cytokines in tears (pg/ml)				
IL-1 β	<1.0	<1.0	<1.0	<1.0
IL-2	<3.0	<3.0	<3.0	<3.0
IL-3	<31.2	<31.2	<31.2	<31.2
IL-4	1.0 ± 0.3	0.8 ± 0.1	<0.6	0.8 ± 0.2
IL-5	<3.0	3.2 ± 0.2	<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	2.4 ± 0.6	2.7 ± 0.3	2.9 ± 1.0	2.0 ± 0.5
IL-10	3.3 ± 0.2	3.1 ± 0.1	3.2 ± 0.1	<3.0
IL-12p40	<3.9	<3.9	<3.9	<3.9
IL-12p70	<2.1	<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	1.4 ± 0.2	<1.0
GM-CSF	3.4 ± 0.2	3.3 ± 0.1	<3.0	<3.0
G-CSF	<0.8	1.1 ± 0.3	<0.8	<0.8
TNF- α	<6.0	6.4 ± 0.2	<6.0	<6.0
TGF- β	<6.0	<6.0	<6.0	<6.0

SICR=secondary immediate conjunctival response; SLCR=secondary late conjunctival response; SDYCR=secondary delayed conjunctival response; ^a=normal value $4.0\text{--}10.0 \times 10^9/L$; ^b=normal value $<300 \times 10^6/L$; ^c=normal value $<500\text{ IU/mL}$; ^d=normal value $<0.70\text{ U/mL}$; ^e=positive skin response to the relevant allergen (=allergen producing positive nasal and conjunctival or nasal response only); ^f=normal value $\geq 8.0\text{ mg/mL}$.

The detection limits cytokines in pg/mL: IL-1 β (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) and TGF- β (6.0).

(1:1 = our standard concentrations) (Table 2), as recommended by the manufacturer.

response, and the skin induration recorded later than 48 h to be a delayed skin response.^{7–13}

Skin Tests

Skin prick tests with allergenic extracts in concentrations of 500 BU/mL were evaluated after 20 min. The following intracutaneous tests in concentrations of 100–300 BU/mL were evaluated 20 min and 6, 12, 24, 36, 48, 56, 72 and 96 h after the intradermal injection. A skin flare and wheal ($>7.0\text{ mm}$ in diameter) occurring after 20 min was considered to be a positive immediate skin response, the skin infiltration appearing between 6 and 12 h to be a late skin

Nasal Provocation Tests

Nasal challenges with allergens were performed by means of rhinomanometry, described in our previous studies.^{7–12,28} The nasal obstruction due to the nasal mucosa edema was evaluated by means of nasopharynx-nostril pressure gradients (NPG), which are the pressure differences (ΔP) between the nasopharyngeal cavity and the outside air, expressed in cm H₂O. NPTs were performed according to the following schedule: (1) baseline values recorded at 0, 5 and 10 min; (2) PBS

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

Allergen	Concentration BU/mL	Nasal responses positive (n = 47)	SAC (n = 21)	PAC (n = 26)	Conjunctival responses			
					Positive (n = 47)			Negative (n = 51)
					SICR (n = 16)	SLCR (n = 20)	SDYCR (n = 11)	
<i>Dermatophagoides pteron</i>	1000	5		5	2	2	1	4
<i>Dermatophagoides farinae</i>	1000	1		1	0	1	0	2
Animal danders								
dog	3000	3		3	2	1	0	5
cat	2000	4		4	1	2	1	3
guinea pig	2000	1		1	0	1	0	1
hamster	2000	2		2	1	0	1	2
cow	2000	1		1	0	1	0	0
horse	1000	1		1	1	0	0	0
Feather								
canary	3000	1		1	1	0	0	1
parrot	3000	3		3	0	2	1	2
parakeet	2000	1		1	0	0	1	2
<i>Aspergillus fumigatus</i>	1000	2		2	0	1	1	2
<i>Aspergillus niger</i>	1000	1		1	0	1	0	3
Pollen								
grass mix I	1000	5	5		2	2	1	3
grass mix II	1000	2	2		1	1	0	6
flower mix	5000	2	2		1	0	1	3
tree mix	3000	3	3		1	1	1	4
birch	1000	5	5		1	2	2	3
willow	1000	2	2		1	1	0	2
hazel	1000	1	1		1	0	0	0
poplar	1000	1	1		0	1	0	3

BU/mL = biologic units per millilitre; SAC = seasonal 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* and *Poa pratensis*; Grasspollen mix II = *Festuca pratensis*, *Holcus lanatus*, *Agrostis alba* and *Anthoxanthum odoratum*; Flower pollen mix = *Dahlia variabilis*, *Solidago virgaurea*, *Primula variabilis* and *Forsythia suspensa* and Tree pollen mix = *Betula pendula*, *Corylus avellana*, *Juniperus communis* and *Salix alba*.

control values recorded at 0, 5 and 10 min after a three-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 three-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 min, then every hour up to the 12th hour, and every second hour during the time-periods between the 24th–38th and 48th–56th (60th) h.^{7–13} The allergens used for the NPTs were chosen with respect to the disease history and/or skin tests (Tables 1 and 2).

All NPTs were performed with allergenic extracts in 1:5 dilution and if they were negative, then with extracts in 1:1 dilution (our basic concentrations) (Table 2). The nasal response was assessed to be positive when the post-challenge mean 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.^{7–13} The NPG changes recorded within 60–120 min after the allergen challenge were considered to be an immediate (INR), those recorded within 4–12 h to be a late (LNR) and

the changes measured later than 24 h to be a delayed (DYNR) response.^{7–14,20,21,29}

Control Challenge with PBS

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

Conjunctival Response (CR)

The objective conjunctival signs and subjective symptoms were registered before and during all NPTs with allergens and PBS controls at the same time-points as the nasal NPG values. The conjunctiva was assessed by ophthalmoscopy including a slit lamp.

The conjunctival signs, hyperaemia (injection), chemosis, hyperlacrimation and palpebral edema, and the subjective symptoms, itching (burning), blurred vision and photophobia, were evaluated by means of the scale suggested by Abelson et al.,^{31,32} however, modified by the author (Pelikan's scale).^{9,10,12,13,29}

Collection and Processing of Tears

The tear specimens were collected from the inferior conjunctival fornix and/or lacus lacrimalis of each of the eyes separately by means of a micropipette at the following time-intervals: (a) before the NPT: one mixed sample of tears collected at 10, 5 and 0 min, (b) after the NPT: mixed tear samples 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 24th–38th and 48th–56th (60th) h.

Determination of Cytokines in Tears

The tear samples (0.1–0.4 mL) were stored at -10°C and processed within 1 h. After a centrifugation at $2000 \times g$ at 4°C for 5 min, the cytokines were determined in supernatants by commercial immunoassay (enzyme-linked immunosorbent assay [ELISA] and enzyme immunoassay [EIA]) kits, according to the manufacturer's recommendations. The measurements were performed in tear samples from each eye separately on each occasion, applying a double-blind schedule. The results were calculated as the mean of both the eyes.

The detection limits 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), tumor necrosis factor alpha (TNF- α) (6.0) and TGF- β (6.0) were measured by means of R&D System (Minneapolis, MN) kits; IL-4 (0.6), IL-8 (1.3), IL-12p70 (2.1), IL-18 (9.2) and interferon gamma (IFN- γ) (1.0) by Bender MedSystems (Wien, Austria) and IL-12p40 (3.9) by Becton Dickinson (San Jose, CA) kits. The inter-assay as well as intra-assay coefficients of variations for these kits were less than 10%.

Control Group

In 14 allergic rhinitis patients with normal ophthalmologic findings volunteering to participate as control subjects, 14 positive NPTs with inhalant allergens (five INR, six LNR and three DYANR) were repeated and supplemented with recording of the conjunctival and subjective symptoms and single pre-challenge determination of the cytokines in tears.

Statistical Analysis

The initial and repeated nasal and the ocular responses (mean total symptom scores) and the PBS controls in individual patients were statistically evaluated by generalized multivariate analysis of

variance model (MANOVA).³³ The polynomials were fitted to the mean curves over time (8 time points within 120 min and 25 time points up to 72 h after the allergen challenge), and the hypotheses were tested by the modified MANOVA computerized system.³³

The post-challenge 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 pre-challenge values and analyzed by Wilcoxon matched-pair signed rank test.

The mean post-challenge cytokine values measured at each of the time points during the repeated NPTs were also compared with corresponding PBS values and evaluated by Mann–Whitney *U* test. Statistical evaluation was performed separately for each of the eyes, and the mean from both the eyes was then calculated. A *p* value <0.05 was considered to be statistically significant.

RESULTS

Nasal Responses (NR)

In the 47 patients, the 98 NPTs with various inhalant allergens (Table 2) and 47 PBS control challenges were performed. They developed 47 positive nasal responses, 16 immediate (INR; $p < 0.01$), 20 late (LNR; $p < 0.01$), 11 delayed (DYNR; $p < 0.05$) and 51 negative nasal responses (NNRs; $p > 0.1$) to the NPTs with allergen concentrations of 1:1 (Table 2). The NPTs with allergen concentrations of 1:5 were negative ($p > 0.05$). The 47 PBS control tests were negative ($p > 0.2$). No significant differences were found in the development of particular NR types with respect to the individual allergens ($p > 0.2$). The repeated NPTs resulted in the development of the similar NR types (Figures 1C, 2C, and 3C). The differences between the initial and the repeated responses were statistically not significant ($p > 0.1$).

Conjunctival Responses

The 47 positive NRs were associated with significantly positive SCRs, 16 immediate (SICR; $p < 0.01$), 20 late (SLCR; $p < 0.001$) and 11 delayed (SDYCR; $p < 0.05$). No SCR were observed during the 51 NNRs ($p > 0.1$).

The SCRs were characterized by significant changes of the objective conjunctival signs ($p < 0.001$) and subjective symptoms ($p < 0.05$). The 47 repeated NPTs, have induced similar and statistically significant SCRs, both as comparing the post-challenge with the pre-challenge (baseline) values ($p < 0.01$) and as comparing with the PBS control challenge ($p < 0.01$) (Figures 1B, 2B and 3B). Neither were significant

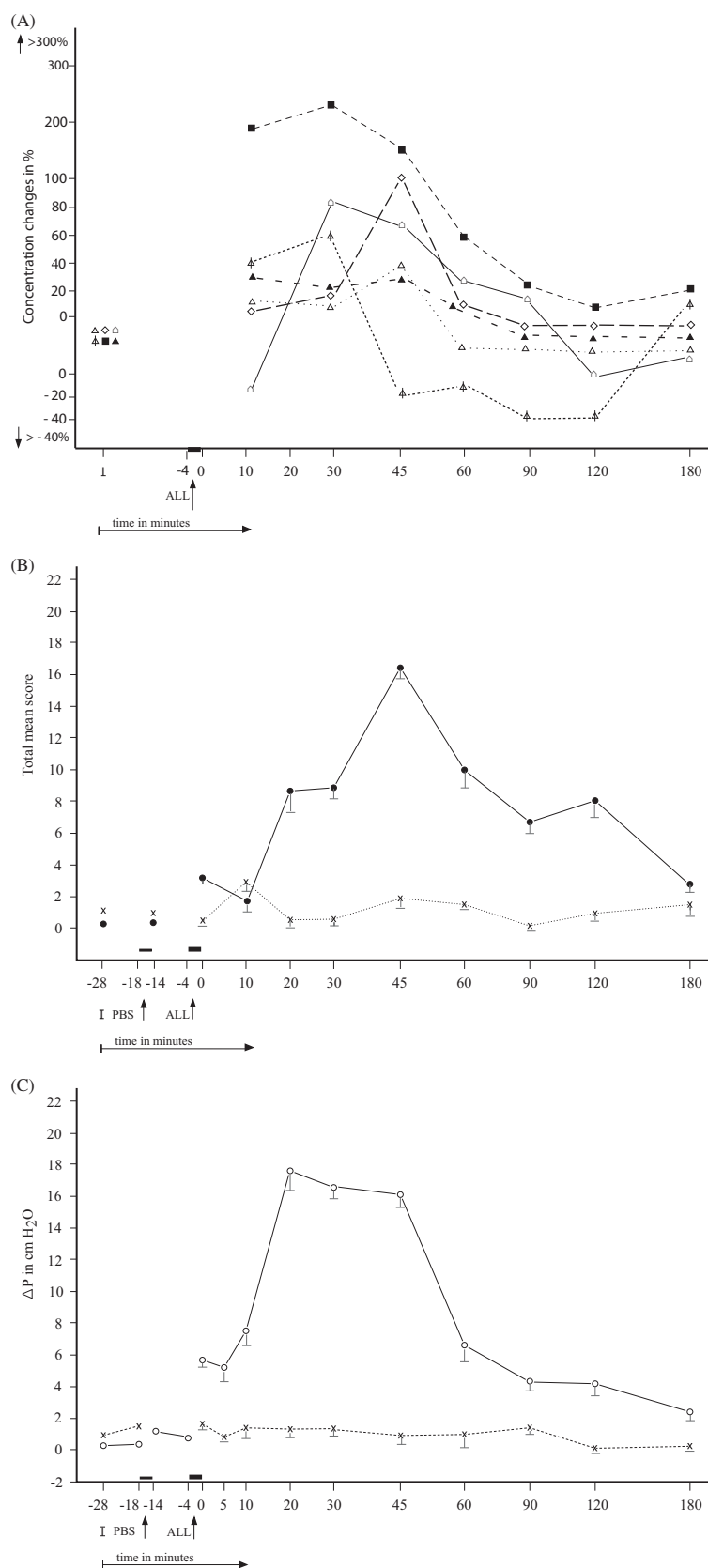


FIGURE 1 The secondary immediate conjunctival responses (SICRs; $n=16$) (A) The mean score of particular factors during the SPCR: Δ = Interleukin-3, $\blacksquare$ = Interleukin-4, $\blacktriangle$ = Interleukin-5, $\blacklozenge$ = Interleukin-8, $\diamond$ = Interleukin-10, $\triangle$ = granulocyte-macrophage colony-stimulating factor. (B) The total mean score of conjunctival signs and symptoms during the SPCR ($\bullet$) and PBS (x). (C) The mean rhinomanometric values (NPG) recorded during immediate nasal response ($\circ$) and PBS (x). I=Initial (baseline) values; PBS=phosphate buffered saline; ALL=allergen.

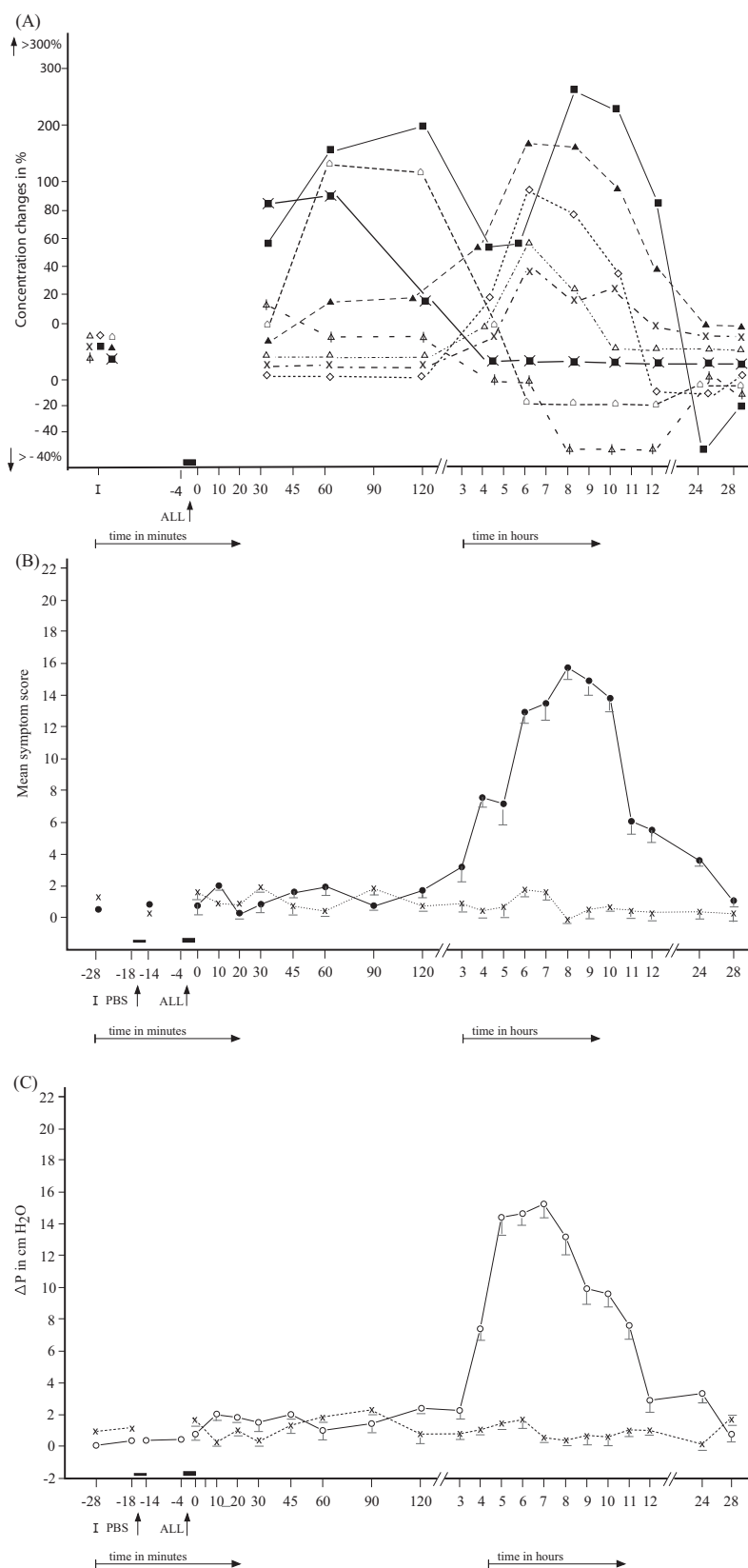


FIGURE 2 The secondary late conjunctival responses (SLCRs; $n=20$). (A) The mean score of particular factors during the SLCR: Δ = interleukin-3, $\blacksquare$ = interleukin-4, $\blacktriangle$ = interleukin-5, $\blacktriangleup$ = interleukin-8, $\diamond$ = interleukin-10, $\blacksquare\times$ = interleukin-12p40, $\triangle$ = granulocyte-macrophage colony-stimulating factor, $\times$ = granulocyte colony-stimulating. (B). The total mean score of conjunctival signs and symptoms during the SLCR (●) and PBS (x). (C) The mean rhinomanometric values (NPG) recorded during late nasal response (○) and PBS (x). I = initial (baseline) values; PBS = phosphate buffered saline; ALL = allergen.

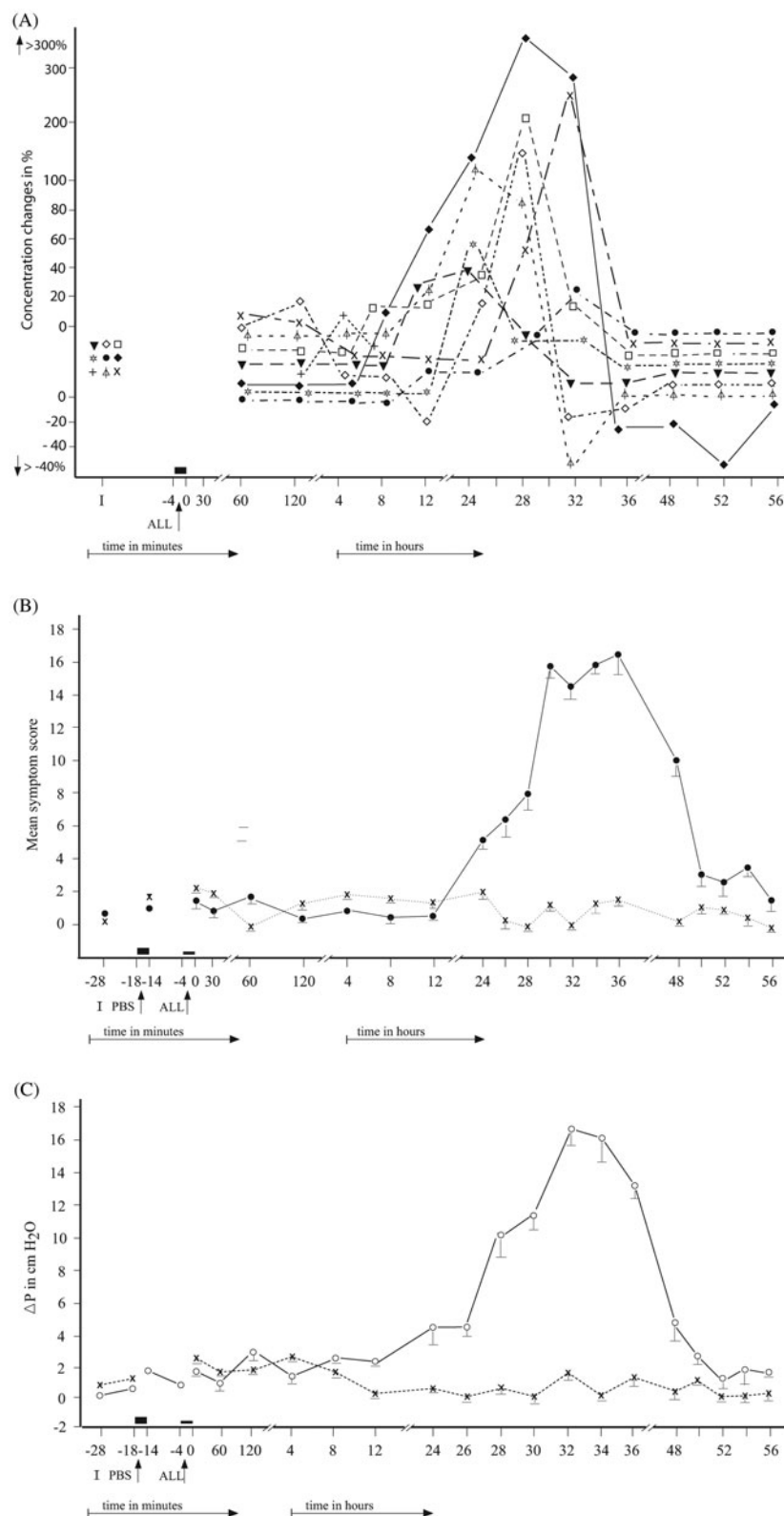


FIGURE 3 The secondary delayed conjunctival responses (SDYCRs; $n=11$). (A) The mean score of particular factors during the SDYCR: □ = interleukin-2, * = interleukin-7, ▲ = interleukin-8, ◇ = interleukin-10, + = interleukin-18, ◆ = interferon- γ , ▼ = tumor necrosis factor- α , x = granulocyte colony-stimulating factor, ● = transforming growth factor- β . (B) The total mean score of conjunctival signs and symptoms during the SDYCR (●) and PBS (x). (C) The mean rhinomanometric values (NPG) recorded during delayed nasal response (○) and PBS (x). I = initial (baseline) values; PBS = phosphate buffered saline; ALL = allergen.

TABLE 3 Significant changes in concentration of particular cytokines in tears during the secondary immediate conjunctival response (SICR; $n = 16$).

	Before the challenge	After the challenge (minutes)							
		0–10	20–30	45	60	90	120	180	240
IL-3 (pg/mL)									
SICR	31.4 ± 0.1	33.7 ± 0.5	32.8 ± 0.6	43.1 ± 1.5*	32.5 ± 0.7	31.2 ± 0.1	32.1 ± 0.8	32.0 ± 0.5	<31.2
PBS	1.5 ± 0.4	<31.2	31.7 ± 0.3	32.9 ± 0.6	32.0 ± 0.5	<31.2	<31.2	31.4 ± 0.2	31.3 ± 0.2
IL-4 (pg/mL)									
SICR	0.9 ± 0.2	2.8 ± 0.7*	3.0 ± 0.8*	2.2 ± 0.9*	1.5 ± 0.6	1.1 ± 0.4	1.0 ± 0.3	1.1 ± 0.7	1.0 ± 0.4
PBS	0.9 ± 0.3	1.2 ± 0.3	1.2 ± 0.6	0.8 ± 0.2	1.3 ± 0.5	1.0 ± 0.1	1.2 ± 0.5	1.4 ± 0.5	0.8 ± 0.2
IL-5 (pg/mL)									
SICR	<3.0	3.9 ± 0.6	3.6 ± 0.3	4.3 ± 0.8 ⁺	3.5 ± 0.4	3.2 ± 0.2	<3.0	<3.0	>3.0
PBS	<3.0	<3.0	<3.0	3.1 ± 0.1	<3.0	<3.0	3.2 ± 0.1	<3.0	<3.0
IL-10 (pg/mL)									
SICR	3.2 ± 0.1	3.4 ± 0.3	3.8 ± 0.6	6.5 ± 1.0*	3.5 ± 0.4	3.4 ± 0.1	3.3 ± 0.2	<3.0	3.2 ± 0.1
PBS	3.4 ± 0.2	<3.0	<3.0	<3.0	3.3 ± 0.2	<3.0	3.0 ± 0.0	<3.0	<3.0
GM-CSF (pg/mL)									
SICR	3.3 ± 0.2	<3.0	6.1 ± 1.3*	5.5 ± 2.6*	4.1 ± 0.6	3.9 ± 0.5	3.1 ± 0.1	3.4 ± 0.3	3.2 ± 0.2
PBS	3.2 ± 0.1	3.5 ± 0.4	3.9 ± 0.5	3.0 ± 0.0	3.2 ± 0.1	3.3 ± 0.2	3.5 ± 0.7	3.1 ± 0.1	3.3 ± 0.3

SICR = secondary immediate conjunctival response; PBS = phosphate-buffered saline; Significance with respect to the baseline (before the challenge): ⁺ = $p \leq 0.05$; * = $p < 0.05$ (in bold).

differences in the conjunctival features between the right and left eye recorded during the initial and the repeated SCRs ($p > 0.1$). No significant corneal signs were recorded in any SCR type. No conjunctival changes were recorded during the 47 PBS control challenges ($p > 0.1$) or during the 51 NNRs ($p > 0.1$).

Changes of the Cytokines in the Tears During the SCRs

The SICRs were associated with significant concentration changes ($p < 0.05$) of IL-3, IL-4, IL-10 and GM-CSF in the tears (Table 3; Figure 1A). The SLCRs were accompanied by significant changes in the concentrations ($p < 0.05$) of IL-3, IL-4, IL-5, IL-8, IL-10, IL-12p40, GM-CSF and G-CSF (Table 4; Figure 2A). The SDYCRs were associated with significant concentration changes ($p < 0.05$) of IL-2, IL-8, IL-10, IFN- γ , G-CSF and TNF- α in the tears (Table 5; Figure 3A). The other cytokines studied did not demonstrate any significant concentration changes in tears during the particular types of the SCR or their concentrations in tears were under the detection limits.

The pre-challenge concentrations of most of the cytokines in tears were very low or under the detection limit and did not differ significantly from those measured in the healthy control subjects (Table 1). No significant concentration changes of these cytokines were recorded in tears during the 47 PBS controls ($p > 0.1$) or 51 negative CRs ($p > 0.2$). No significant differences in the concentration changes of the particular cytokines in tears have been found between the right and left eye, both

during the SCRs and during the PBS controls ($p > 0.1$ and $p > 0.2$, respectively).

Control Patients

The 14 control subjects did not demonstrate any conjunctival signs or subjective ocular symptoms during the 14 repeated positive nasal responses ($p > 0.1$, $p < 0.05$, respectively). Their pre-challenge cytokine concentrations in tears are presented in Table 1.

DISCUSSION

The existence of two basic forms of AC, the primary AC and the secondary AC form, induced by allergic event in the nasal mucosa, may have a significant impact on the therapeutic management of this disorder.^{7–13,29} Although the possible causal role of nasal allergy in some AC cases was identified by us more than 20 years ago,¹¹ it has, unfortunately, not sufficiently attracted the attention of the relevant clinicians.^{1–6}

The relationship between the conjunctivae and the nasal cavity is implemented both at the anatomical and the functional level.^{1–13} The link between both the organs includes (a) connection of the conjunctiva with the nasal cavity through the naso-lacrimal duct, executing the tear drainage into nasal cavity and allowing, under certain conditions, also a retrograde penetration of the cells and various factors, released by and in the nasal mucosa, into the conjunctivae; (b) communication of both the systems through the local blood vessel, lymphatic and neurogenic

TABLE 4 Significant changes in concentration of particular cytokines in tears during the secondary late conjunctival response (SLCR; $n = 20$).

		After the challenge (hours)									
Before the challenge		½	1	2	4	6	8	10	12	24	28
IL-3 (pg/mL)											
SLCR	<31.2	<31.2	31.7 ± 0.5	<31.2	<31.2	49.7 ± 1.8*	42.5 ± 0.7*	32.2 ± 0.5	<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 (pg/mL)											
SLCR	1.3 ± 0.5	2.1 ± 0.6	3.5 ± 0.9*	3.9 ± 1.2*	2.0 ± 0.6	2.1 ± 0.8	4.8 ± 2.4*	4.5 ± 0.7*	2.4 ± 0.7	<0.6	1.1 ± 0.1
PBS	0.9 ± 0.3	1.4 ± 0.3	1.2 ± 0.6	1.1 ± 0.5	1.3 ± 0.3	1.0 ± 0.1	1.3 ± 0.2	1.4 ± 0.3	<0.6	<0.6	0.8 ± 0.2
IL-5 (pg/mL)											
SLCR	3.4 ± 0.1	3.6 ± 0.4	4.0 ± 1.0	4.1 ± 0.8	5.5 ± 1.3	9.7 ± 1.3*	9.4 ± 1.0*	6.8 ± 0.6*	4.6 ± 1.3	3.5 ± 0.4	3.2 ± 0.1
PBS	3.3 ± 0.2	3.1 ± 0.1	3.3 ± 0.3	3.1 ± 0.1	3.2 ± 0.1	<3.0	<3.0	<3.0	3.1 ± 0.1	<3.0	3.3 ± 0.2
IL-8 (pg/mL)											
SLCR	2.5 ± 0.9	2.8 ± 0.6	2.5 ± 0.5	2.7 ± 0.4	2.6 ± 0.1	2.6 ± 0.3	<1.3*	<1.3*	<1.3 *	2.4 ± 1.0	1.8 ± 0.3
PBS	2.2 ± 0.5	1.5 ± 0.2	1.9 ± 0.4	2.0 ± 0.5	2.1 ± 0.8	1.7 ± 0.3	2.2 ± 0.9	1.4 ± 0.1	1.7 ± 0.3	2.0 ± 0.5	2.0 ± 0.4
IL-10 (pg/mL)											
SLCR	3.2 ± 0.2	3.4 ± 0.3	3.5 ± 0.7	3.1 ± 0.1	3.8 ± 0.6	6.1 ± 1.0*	5.5 ± 1.5*	4.4 ± 1.2	<3.0	<3.0	3.3 ± 0.2
PBS	3.1 ± 0.1	<3.0	<3.0	<3.0	3.3 ± 0.2	<3.0	3.0 ± 0.0	<3.0	<3.0	<3.0	<3.0
IL-12p40 (pg/mL)											
SLCR	<3.9	7.3 ± 1.1*	7.5 ± 1.4*	4.6 ± 0.5	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9
PBS	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9	<3.9
GM-CSF (pg/mL)											
SLCR	3.6 ± 0.3	3.7 ± 0.3	8.5 ± 2.6*	8.1 ± 1.3*	3.9 ± 1.0	<3.0*	<3.0*	<3.0*	3.4 ± 0.3	3.4 ± 0.2	3.5 ± 0.3
PBS	3.5 ± 0.5	3.2 ± 0.1	3.9 ± 0.5	3.5 ± 0.1	3.2 ± 0.2	3.9 ± 0.4	3.5 ± 0.5	3.7 ± 0.3	3.0 ± 0.0	3.2 ± 0.1	3.3 ± 0.2
G-CSF (pg/mL)											
SLCR	<0.8	1.5 ± 0.6	<0.8	<0.8	<0.8	3.7 ± 1.3*	3.9 ± 0.8*	1.0 ± 0.4	<0.8	<0.8	<0.8
PBS	<0.8	<0.8	<0.8	<0.8	1.0 ± 0.2	0.9 ± 0.1	<0.8	<0.8	<0.8	<0.8	<0.8
TNF-α (pg/mL)											
SLCR	6.7 ± 0.4	6.2 ± 0.1	6.7 ± 0.5	7.0 ± 1.0	7.8 ± 0.7 ⁺	6.9 ± 0.6	6.8 ± 0.3	7.1 ± 0.8	6.4 ± 0.3	6.9 ± 0.5	6.6 ± 0.4
PBS	6.1 ± 0.1	<6.0	6.6 ± 0.5	6.9 ± 0.8	<6.0	<6.0	<6.0	6.5 ± 0.4	6.3 ± 0.2	6.0 ± 0.0	<6.0

SLCR = secondary late conjunctival response; PBS = phosphate-buffered saline; Significance with respect to the baseline (before the challenge): ⁺ = $p \leq 0.05$; * = $p < 0.05$ (in bold).

network.¹⁻¹⁰ The allergic reactions taking place primarily in the nasal mucosa due to the intranasal exposure to the external, mostly inhalant, allergens can affect the conjunctivae in various ways and upon the involvement of a number of primary as well as secondary mechanisms and modalities, such as (1) the factors, such as various mediators, constituents, cytokines and chemokines released by the allergic reaction in the nasal mucosa could reach the conjunctiva either indirectly having been transported by the local blood vessels, such as *arteriae maxillaris – pars pterygo – palatina*, *vena facialis*, *plexus pterygoideus* or probably directly by the retrograde penetration through the naso-lacrimal duct; (2) the cells participating directly in the nasal allergic reactions or those having been released from the nasal mucosa during the allergic reaction, can migrate either through the blood and/or lymphatic system into the conjunctival tissue or into the nasal secretions and, under some conditions, migrate through the naso-lacrimal duct into the conjunctivae;⁸⁻¹⁰ (3) the allergic reactions occurring 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 the nose-associated lymphatic tissue, communicating with various eye-related lymphatic tissues, such as tear duct-associated lymphatic tissue, lacrimal drainage-associated lymphatic tissue, conjunctiva-associated lymphatic tissue and eye-associated lymphatic tissue.^{8-10,34-37} Through the manifold communication network among the particular lymphatic tissues, various intracellular as well as the cell membrane-bound receptor signals can be transmitted and the traffic of various cell types between the related and/or adjacent tissues can be realized;^{8-10,34-37} (5) the so-called “defective homing mechanism” can also participate in the communication between the nasal mucosa and conjunctival tissue. Cell traffic is realized not only by means of attraction mechanisms, governed by chemotactic factors, cytokines, lymphokines and adhesion molecules, but also through the so-called “homing” mechanism of the B- and T-lymphocytes, controlled by a number of homing factors. Sub-sets of lymphocytes initially activated in one tissue, migrating then into the blood stream and/or lymphatic network to finish their maturation process, do not return to this original tissue, but due to the disturbed homing factors they migrate into other tissues in different localities;^{8-10,37}

TABLE 5 Significant changes in concentration of particular cytokines in tears during the secondary delayed conjunctival response (SDYCR; $n = 11$).

		After the challenge (hours)										
Before the challenge		12	24	26	28	30	32	34	36	48	52	56
IL-2 (pg/mL)												
SDYCR	<3.0	3.5 ± 0.4	3.9 ± 0.6	7.8 ± 1.0*	9.9 ± 1.4*	4.2 ± 1.1	3.3 ± 0.3	<3.0	<3.0	<3.0	<3.0	<3.0
PBS	<3.0	3.0 ± 0.0	3.2 ± 0.1	3.1 ± 0.1	<3.0	<3.0	<3.0	3.3 ± 0.2	<3.0	<3.0	<3.0	<3.0
IL-8 (pg/mL)												
SDYCR	2.5 ± 0.8	3.1 ± 1.0	5.9 ± 0.7*	3.6 ± 0.5	3.6 ± 1.0	<1.3*	<1.3*	<1.3*	2.5 ± 0.4	2.6 ± 0.5	2.4 ± 0.3	2.5 ± 0.6
PBS	2.3 ± 0.4	2.5 ± 0.7	2.1 ± 0.2	2.0 ± 0.3	2.1 ± 0.9	1.7 ± 0.5	2.2 ± 0.8	2.4 ± 0.9	1.8 ± 0.5	1.9 ± 0.2	1.4 ± 0.1	2.0 ± 0.5
IL-10 (pg/mL)												
SDYCR	3.7 ± 0.2	3.0 ± 0.3	4.5 ± 1.0	4.1 ± 0.8	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 (pg/mL)												
SDYCR	1.5 ± 0.5	2.5 ± 1.0	3.8 ± 0.5 ⁺	8.7 ± 2.3*	7.9 ± 2.0*	7.5 ± 2.3*	5.8 ± 0.4*	2.0 ± 1.4	1.1 ± 0.4	1.2 ± 0.5	<1.0	1.5 ± 0.3
PBS	1.3 ± 0.2	1.4 ± 0.3	1.5 ± 0.4	1.8 ± 0.6	1.0 ± 0.0	2.0 ± 0.1	2.2 ± 1.0	1.8 ± 0.5	1.6 ± 0.5	1.5 ± 0.3	1.7 ± 0.6	1.5 ± 0.5
G-CSF (pg/mL)												
SDYCR	<0.8	<0.8	<0.8	<0.8	1.2 ± 0.3	3.0 ± 1.0*	2.8 ± 0.7*	2.0 ± 0.6*	<0.8	0.9 ± 0.1	<0.8	<0.8
PBS	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8
TNF-α (pg/mL)												
SDYCR	<6.0	8.0 ± 1.6*	9.5 ± 1.0*	7.5 ± 1.0	<6.0	<6.0	<6.0	6.2 ± 0.1	<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 (pg/mL)												
SDYCR	<6.0	<6.0	<6.0	<6.0	<6.0	8.6 ± 1.3 ⁺	8.5 ± 1.1 ⁺	7.8 ± 0.9	<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; PBS = phosphate-buffered saline; Significance with respect to the baseline (before the challenge): ⁺ = $p < \pm 0.05$; * = $p < 0.05$ (in bold).

(6) finally, some additional factors and mechanisms could, under some circumstances, influence the relation of nasal mucosa and conjunctiva, such as increased intranasal pressure due to edematic nasal mucosa and disturbed air passage through the nose and disturbance or partial stagnation of the tear drainage through the ductus nasolacrimalis.

Various immunologic mechanisms, such as immediate (IgE-mediated, Type I), late (Type III) or delayed (cell-mediated, Type IV) can be involved in the primary AC forms as well as in the secondary (induced) AC forms.^{1–24,38} Different hypersensitivity mechanisms result then in the development of the different types of primary CR to the direct exposure of the conjunctiva to external allergens, such as an immediate/early (ICR), late (LCR) or delayed (DYCR) response.^{1–22}

Analogically, different hypersensitivity mechanisms participating in the nasal mucosa can lead to the development of different types of nasal response, inducing then different types of the SCR, such as an SICR, SLCR or SDYCR responses.^{7–13}

Occurrence of cytokines in tears of patients suffering from AC has already been investigated.^{15–18,22,23,25–28} The following cytokines have been detected in the tears of patients suffering from primary forms of AC: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IFN- γ and TNF- α .^{1,3,10,15–23,25–29} However, in most of these studies, only a single determination of the cytokines in tears has been

performed and the results vary. Unfortunately, data concerning the concentration changes of particular cytokines in tears during the individual types of the primary form of CR to the CPTs with allergens are lacking in the literature.

Moreover, little data are also available on the cytokine profiles in the tears of the healthy subjects, which may serve as reference values.^{39,40} Finally, because the appearance of cytokines in tears during the secondary forms of CR to NPTs has not yet been investigated, our results cannot be compared with other data.

Our results demonstrating significant concentration changes ($p < 0.05$), mostly an increase of IL-3, IL-4, IL-10 and GM-CSF in tears during the SICR indicate an involvement of IgE-mediated hypersensitivity upon participation of mast cells, eosinophils and Th₂-lymphocytes in the mechanism underlying this response type. The significant concentration changes of IL-3, IL-4, IL-5, IL-8, IL-10, IL-12p40, GM-CSF and G-CSF in tears during the SLCR would suggest the participation of basophils, eosinophils, neutrophils and Th₂-lymphocytes in SLCR. The concentration changes of IL-2, IL-8, IL-10, IFN- γ , G-CSF and TNF- α in tears during the SDYCR are indicative of an involvement of neutrophils, monocytes and Th₁-lymphocytes in this response type. However, the very low cytokine concentrations in tears recorded during the secondarily induced CR types, as compared with cytokine concentrations during the primary AC

forms, would suggest their probable origin in the nasal mucosa, where they had been released during the primary nasal allergic reactions. This hypothesis is supported by our previous results, demonstrating only very limited numbers of non-activated cells in tears during the SCR types.^{7-10,12,13} Nevertheless, to distinguish exactly the basic portion of tears related to the genuine immunologic mechanism from that portion being caused by reflex mechanisms is a very difficult if not impossible task. Nevertheless, we are convinced that the tear specimens, collected by the very careful and non-irritating technique used in this study, have been produced by the factors released by a genuine immunologic mechanism(s) initiated in the nasal mucosa. On the other hand, the lack of recording of cytokines in the nasal secretions simultaneously to their recording in tears, which had not been performed for technical reasons, may be qualified as a certain deficit of the current study. This aspect will certainly be included in the next studies.

In addition, the results of this study demonstrate also the diagnostic value of provocation tests with allergens in some of the AC patients. The CPTs with allergens performed directly on the conjunctiva, confirm the role of allergic reaction occurring in the conjunctiva due to a direct exposure of conjunctival tissue to an inhalant allergen.^{6,14,18,20,21,23,29,30,32,41-44} The CPTs resulting in the manifestation of various types of primary CR CPTs are therefore suitable for demonstrating the primary types of CR.^{6,14,18,20,21,23,29,30,32,41-44} In contrast, the particular CR types 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.⁷⁻¹³

The results of this study are, however, limited only to the tears and they cannot indicate the histological changes and processes in the conjunctival tissue during the particular types of the SCRs. The histologic changes in the conjunctival tissue can only be demonstrated by repeated impression cytology and/or biopsies, combined eventually with flow-cytometry. Unfortunately, the histological changes in conjunctival tissue were not investigated with respect to some technical reasons. This fact may be regarded as a limitation of this study. The histological changes in the conjunctival tissue during the particular types of the SCRs will be investigated in the following studies.

The results of this study demonstrated unequivocally existence of the various types of the SCR induced by the nasal allergy, which were associated with different cytokine profiles in the tears suggesting involvement of different hypersensitivity mechanisms in these particular CR types. These results emphasize not only the diagnostic value of NPTs with allergens combined with monitoring of various ocular features and factors in tears in patients suffering from the secondary AC forms, but they also may have an

impact on the therapeutical approach to this clinical entity, especially in AC patients who did not respond satisfactorily to the usual ophthalmologic treatment. Results of some of our pilot studies performed in the past have demonstrated distinctly increased protective effects of a combination of intraconjunctival and intranasal Cromolyn in the patients with SCRs, especially in those developing the immediate and the late response types.^{8,11,45-47}

However, an indispensable requirement for both of the provocation tests is the registration of the representative parameters before and repeatedly after the allergen challenge for a sufficiently long period of time, allowing the investigator to follow 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 investigation will be needed to clarify exactly the immunologic mechanism(s) underlying the primary as well as the SCR types.

DECLARATION OF INTEREST

Author has no conflict of interests to be disclosed.

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