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Conjunctival provocation with airborne allergen in patients with atopic keratoconjunctivitis

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Summary

Background Atopic keratoconjunctivitis (AKC) is a chronic eye disease with periods of exacerbations. Many patients experience no obvious seasonal variation, although a majority of patients are allergic to common airborne allergens.

Objective To investigate the allergic reaction, to conjunctival provocation with airborne allergens, in patients with AKC.

Methods Eleven patients with AKC and birch and/or grass pollen allergy participated in the study, which was performed outside the pollen season. Five patients with seasonal allergic conjunctivitis (SAC) and five healthy subjects were included for validation purposes. The challenge was performed in one eye with the allergen, to which the patient was reactive, and with dilution buffer in the other eye. Signs and symptoms from both eyes were graded at baseline and at 10 min, 8 and 48 h after provocation. Tear fluid was collected from both eyes for cytokine analyses at baseline and at 8 and 48 h.

Results A significant change in clinical symptoms and signs, (redness and chemosis) was evident 10 min after provocation compared with baseline ($P = 0.005$) and compared with the unprovoked eye ($P = 0.005$) in AKC subjects. These parameters were normalized after 8 and 48 h. A significant increase for IFN- γ ($P = 0.021$) and IL-6 ($P = 0.015$), and a near significant increase for IL-10 ($P = 0.066$) were seen in the tear fluid of the challenged eye at 48 h after provocation vs. baseline and vs. the control eye for IFN- γ ($P = 0.005$), IL-6 ($P = 0.028$) and IL-10 ($P = 0.008$) in AKC subjects.

Conclusion and Clinical Relevance In this single dose allergen provocation study, AKC patients responded with a typical IgE-mediated allergic reaction. An increase in cytokines at 48 h after the challenge was demonstrated and might, with further studies, give us a better understanding of the nature of inflammation in AKC.

Keywords atopic keratoconjunctivitis, cytokine, eye, IgE, pollen, provocation, seasonal allergic conjunctivitis, tear

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Introduction

The role of allergic sensitization in atopic keratoconjunctivitis (AKC) is unclear and poorly investigated. About 80% of the patients present with an atopic phenotype, defined by IgE antibodies to common allergens, but symptoms and objective parameters of allergic inflammation do not differ between the atopic and the non-atopic subgroups [1–3]. AKC is invariably associated with atopic dermatitis (AD) and practically all AKC patients suffer from eyelid eczema, blepharitis and conjunctivitis and many also from corneal inflammation

[1, 3]. The disease is chronic and normally starts off in young adulthood with periods of exacerbations during the following decades. AKC is a T cell-mediated disease [4–6], but the impact of environmental effects is not fully understood. Even though a majority of AKC patients are allergic to pollen, flare-ups occur all around the year without a typical seasonal pattern [1]. Conjunctival provocation test (CPT) is one way to identify local sensitization to allergens and to explore inflammatory mediators in the allergic response. In seasonal and perennial allergic conjunctivitis, CPT is sometimes used for diagnostic purposes and above all

applied in pharmacodynamic studies [7–9]. As for chronic eye allergy, there are but two published studies [10, 11] describing CPT in vernal keratoconjunctivitis (VKC), and to the best of our knowledge, CPT has previously not been performed in AKC patients. The aim of the study was to gain better knowledge of the possible contribution of pollen allergy to the chronic conjunctivitis in AKC patients. Conjunctival provocations with pollen allergens were performed among AKC patients, symptoms were documented and cytokines were analysed in tear fluid.

Materials and methods

Subjects and study design

Eleven AKC patients (5 women and 6 men; aged 27–66 years; median 46 years) participated in the study. The patients were known to the investigators and had moderate AKC disease with AD and low-grade chronic conjunctival and corneal inflammation and intermittent need of treatment. Inclusion required 2 weeks of wash-out of topical or systemic anti-inflammatory or anti-infective treatment and a positive skin prick test (SPT) to birch or grass pollen (Fig. 1). Five patients with SAC due to birch or grass pollen verified with SPT, but no other eye disease, and five healthy subjects (CT) with no history of allergy or other eye diseases were recruited to provide reference data (Table 1). The study was performed outside the pollen season.

Skin test

The skin test was performed with Soluprick SQ *Betula verrucosa* (silver birch), 10 HEP and Soluprick SQ Grass Pollen *Phleum pratense* (timothy grass), 10 HEP (ALK-Abelló Nordic), according to the manufacturer's instructions. Prick tests were considered positive, if the weal reaction was at least 3 mm, or at least 25% of the

weal area induced by simultaneous pricking with Soluprick positive control. The allergen that gave the greatest response by skin test and that correlated best with seasonal symptoms was chosen for use in the CPT.

Allergen-specific IgE

Venous blood samples were analysed for allergen-specific IgE to birch and timothy grass using the ImmunoCAP® System (Phadia AB, Uppsala, Sweden) following the manufacturer's instructions. The assay had a lower detection limit of 0.10 kU_A/L and an upper limit of 100 kU_A/L, with higher values reported as greater than 100 kU_A/L.

Conjunctival provocation

The challenge was performed with a single dose of 100 000 SQ-E of Aquagen (ALK-Abelló, Nordic; Kungsbacka, Sweden) with either birch or grass pollen in the lower conjunctival sac in one eye and with dilution buffer ALK-Diluent (ALK-Abelló, Nordic) in the other eye (Table 1, Fig. 2). The right eye was chosen for provocation, except in one patient who was provoked in the left eye due to fear of side-effects in her best eye. The patients were informed not to rub the eyes after instillation.

Clinical signs

The same investigator (EN) examined both eyes at every visit. Conjunctival signs of redness and chemosis were assessed using a 4-grade score system (0 = no signs at all; 1 = mild; 2 = moderate and 3 = severe signs). Baseline data were documented and both eyes were graded at 10 min, 8 and 48 h after provocation. In terms of subjective symptoms, the patients were instructed to score their symptoms with a visual analogue scale (VAS) graded 0–10; 0 corresponding to no discomfort and 10 to severe discomfort; this was done for both eyes at the same time-points as described above. A total symptom score was calculated adding the conjunctival scores to the subjective symptoms.

Cytokines in tear fluid

Tear fluid was collected from both eyes before provocation, and after 8 and 48 h. The tear sampling was performed by two investigators and could thereby be performed in both eyes at the same time. A tear volume of at least 30 µL was collected from the external cantus with a glass blood capillary tube (50 µL) and then transferred to Eppendorff tubes and stored at –80°C for cytokine analysis. The concentrations of IFN-γ, TNF-α, IL-5, IL-6, IL-10 and IL-13 were analysed in 1 : 1

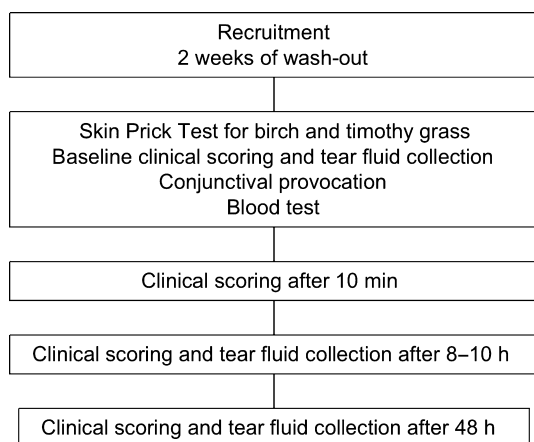


Fig. 1. Study design.

Table 1. Characteristics of patients and control groups

Subject	Gender	Age	Symptoms	Treatment	Weal reaction timothy (mm)	Weal reaction birch (mm)	IgE timothy (kU _A /L)	IgE birch (kU _A /L)	Allergen used for challenge
<i>Atopic keratoconjunctivitis</i>									
1	M	46	Chronic	T	7	8	10	37	Birch
2	M	27	Chronic	S + A	7	10	58	>100	Birch
3	M	47	Chronic	S + A	5	4	6.5	2	Timothy
4	M	49	Chronic	S	4	5	0.62	5.7	Birch
5	M	39	Chronic	T	2	8	<0.1	3.7	Birch
6	F	46	Chronic	A	2	6	31	>100	Birch
7	M	51	Chronic	S + A	12	5	22	41	Timothy
8	F	41	Chronic	A	5	6	0.38	11	Birch
9	F	53	Chronic	S + A	4	0	4	1.4	Timothy
10	F	40	Chronic	T + A	0	4	<0.1	1.0	Birch
11	F	66	Chronic	S	0	5	<0.1	0.42	Birch
<i>Seasonal allergic conjunctivitis</i>									
12	M	47	Seasonal	Seasonal A	4	4	7.9	26	Birch
13	M	36	Seasonal	Seasonal A	8	2	3.2	0.17	Timothy
14	M	22	Seasonal	Seasonal A	9	0	33	0.1	Timothy
15	M	21	Seasonal	Seasonal A	6	3	3.5	0.1	Timothy
16	F	37	Seasonal	Seasonal A	6	8	8.1	50	Birch
<i>Healthy controls</i>									
17	F	50	None	None	0	0	<0.1	<0.1	Birch
18	F	48	None	None	0	0	<0.1	<0.1	Birch
19	F	36	None	None	0	0	<0.1	<0.1	Birch
20	F	63	None	None	0	0	<0.1	<0.1	Birch
21	F	59	None	None	0	0	<0.1	<0.1	Birch

M, male; F, female; T, tacrolimus ointment; S, steroid drops and/or steroid ointment; A, antihistamine drops and/or tablets.

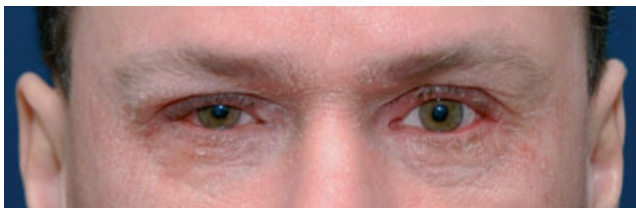


Fig. 2. Atopic keratoconjunctivitis patient no. 1, 10 min after provocation with birch allergen in the right eye and dilution buffer only in the left eye. (Published with permission from the patient).

diluted samples using cytometric bead array (CBA) Flex Set (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Samples were run on a FACSaria flow cytometer and data were analysed using FCAP Array software (BD Biosciences). Detection ranges were 5–10 000 pg/mL for IL-13 and 20–10 000 pg/mL for IFN- γ , TNF- α , IL-5, IL-10 and IL-6.

Ethics

The study followed the Tenets of Declaration of Helsinki. Written informed consent was obtained from each participant and the local ethics committee approved the study.

Statistical analysis

Wilcoxon matched pair test was used to analyse variable differences before and after provocation and between the provoked and unprovoked eye. Correlations were calculated using Spearman's rank correlation coefficient. Differences between the groups were analysed with Mann–Whitney *U*-test. Data were computed with STATISTICA 9 (St. Erik's Eye Hospital, Stockholm, Sweden). A *P*-value of <0.05 was considered statistically significant.

Results

Allergy verification

The included 11 AKC and 5 SAC patients were SPT positive and had IgE antibodies to either birch and/or grass. The healthy control subjects had negative skin prick and IgE tests (Table 1). The allergen-specific IgE levels did not correlate with clinical signs or cytokine levels at baseline or after provocation for either AKC or SAC.

Clinical signs and symptoms

The disease activity in the AKC group was moderate as patients with a more severe disease were unable to halt

topical anti-inflammatory treatment and accordingly could not meet the inclusion criteria. Three of eleven patients scored their symptoms as 7 before the provocation, whereas the median scoring was 4. There was a significant increase in the total symptom scores in the challenged eye 10 min after provocation compared with baseline ($P = 0.005$) (Fig. 3) and with the unprovoked eye ($P = 0.005$) in AKC patients. One patient reported marked discomfort also in the unchallenged eye after 10 min. Both clinical signs and subjective symptoms were normalized at 8 and 48 h after provocation. The same pattern was seen for the SAC subjects, whereas no differences within or between the provoked and unprovoked eye were documented for the CT (Fig. 3). There were no reports of a late-phase reaction with a second flare up of symptoms following the challenge.

Objective parameters of inflammation

The cytokine analysis was performed on one single occasion after completion of the trial. IFN- γ , IL-6, IL-10 and IL-13 were detectable in all study groups, whereas TNF- α and IL-5 were below the detection limit in more than 95% of the samples and therefore excluded from further calculations. Small variations in baseline concentrations between the left and the right eye were seen for IFN- γ , IL-10 and IL-13 in all the study groups. For IL-6, however, there was a significant difference ($P = 0.016$) between the eyes in the AKC patients at baseline.

In AKC patients, no change in cytokine concentration was seen comparing baseline samples with those

collected after 8 h, but after 48 h, an increase was proven for two cytokines: IFN- γ ($P = 0.021$) and IL-6 ($P = 0.015$), whereas the increase for IL-10 nearly reached significance ($P = 0.066$) (Table 2). Moreover, at 48 h, a significant difference was seen between the provoked and the unprovoked eye for the same cytokines: IFN- γ ($P = 0.005$), IL-6 ($P = 0.028$) and IL-10 ($P = 0.008$) (Table 2). In the unprovoked eyes and in the control group, no changes were found after provocation. In SAC patients, only IL-6 was significantly affected with an increase at 48 h after provocation ($P = 0.043$) (Table 2).

There was a positive correlation between the IFN- γ and IL-10 concentrations at all time-points both in the provoked and in the unprovoked eyes in the AKC group: before provocation in both eyes ($r = 0.93$; $P < 0.0001$, in the eyes to be provoked, $r = 0.96$; $P < 0.0001$, in the eye not to be provoked), after 8 h in the provoked eye ($r = 0.78$; $P = 0.005$) and the unprovoked eye ($r = 0.99$; $P < 0.0001$), after 48 h in the provoked eye ($r = 0.86$; $P = 0.001$) and the unprovoked eye ($r = 0.79$; $P = 0.004$). For the other cytokines, occasional correlations were seen with no discernible pattern.

Before provocation, no significant differences in cytokine levels were observed between the AKC, SAC and control groups, but at 48 h after the challenge, higher values in the provoked eyes were detected in AKC patients. Comparing AKC with the healthy controls, the cytokine levels were higher ($P < 0.05$) for all four detectable cytokines. Comparing AKC and SAC

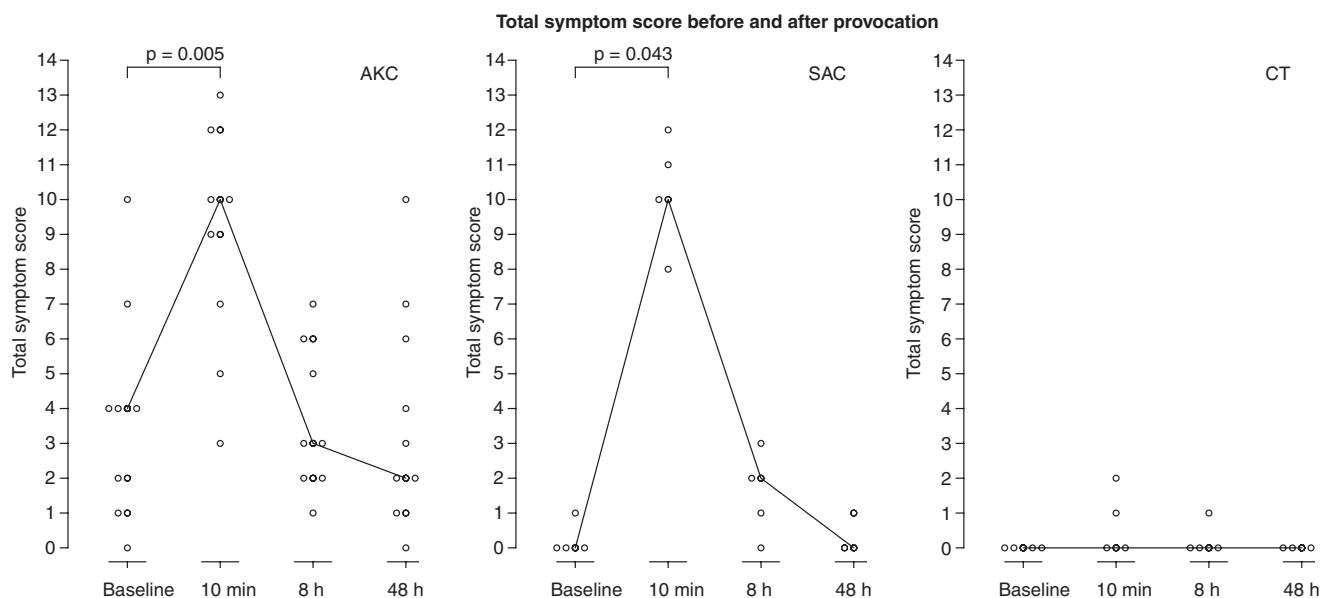


Fig. 3. Total symptom score in the provoked eyes. Median score linked by black line. A significant change from baseline was documented at 10 min for AKC and SAC patients, while no change was seen in healthy controls. AKC, atopic keratoconjunctivitis; SAC, seasonal allergic conjunctivitis; CT, healthy controls.

Table 2. Changes in cytokine levels in AKC, SAC and CT; baseline vs. 48 h after provocation and provoked eye vs. unprovoked eye 48 h after provocation

Subjects	Cytokine	Concentration at baseline (pg/mL) median (25th–75th percentile)	Concentration at 48 h (pg/mL) median (25th–75th percentile)	P-value
AKC	IFN- γ provoked eye	53 (44–63)	88 (73–109)	0.021
AKC	IFN- γ unprovoked eye	77 (59–84)	69 (56–79)	0.328
				0.005*
AKC	IL-6 provoked eye	156 (123–199)	265 (205–341)	0.015
AKC	IL-6 unprovoked eye	194 (144–246)	183 (154–216)	0.756
				0.028*
AKC	IL-10 provoked eye	43 (29–56)	84 (60–96)	0.066
AKC	IL-10 unprovoked eye	69 (52–73)	57 (42–69)	0.859
				0.008*
AKC	IL-13 provoked eye	42 (8–61)	38 (31–68)	0.374
AKC	IL-13 unprovoked eye	50 (10–78)	32 (20–71)	0.878
				0.445*
SAC	IFN- γ provoked eye	42 (36–56)	44 (32–86)	0.892
SAC	IFN- γ unprovoked eye	48 (27–56)	40 (39–51)	0.681
				0.893*
SAC	IL-6 provoked eye	142 (140–145)	154 (141–188)	0.138
SAC	IL-6 unprovoked eye	139 (138–199)	137 (124–156)	0.500
				0.043*
SAC	IL-10 provoked eye	20 (0–32)	0 (0–47)	1.000
SAC	IL-10 unprovoked eye	28 (0–47)	10 (7–38)	0.893
				0.715*
SAC	IL-13 provoked eye	7 (0–23)	11 (0–15)	0.465
SAC	IL-13 unprovoked eye	18 (5–18)	15 (10–32)	0.138
				0.500*
CT	IFN- γ provoked eye	41 (39–47)	36 (33–52)	0.345
CT	IFN- γ unprovoked eye	50 (30–54)	37 (36–45)	0.500
				0.686*
CT	IL-6 provoked eye	86 (70–129)	64 (0–158)	0.138
CT	IL-6 unprovoked eye	77 (46–86)	132 (98–177)	0.225
				0.144*
CT	IL-10 provoked eye	0 (0–31)	0 (0–21)	0.285
CT	IL-10 unprovoked eye	35 (16–38)	0 (0–35)	0.465
				0.593*
CT	IL-13 provoked eye	7 (0–22)	5 (0–26)	1.000
CT	IL-13 unprovoked eye	7 (0–15)	8 (0–19)	0.715
				0.715*

AKC, atopic keratoconjunctivitis; SAC, seasonal allergic conjunctivitis; CT, healthy controls.

*Provoked eye vs. unprovoked eye 48 h after provocation. Bold indicates $P < 0.05$

patients, a significant difference was seen only for IL-6 ($P = 0.017$), borderline significance was found for IFN- γ ($P = 0.058$), while a tendency could be described for IL-10 ($P = 0.076$) and IL-13 ($P = 0.076$). No significant differences between the groups were seen for the ratios: IFN- γ /IL-6, IFN- γ /IL-10 and IFN- γ /IL-13 either before or after provocation.

Discussion

Although a majority of AKC patients are sensitized to airborne allergens, the role of allergen exposure in the disease process remains unclear. There are no longitudinal

observational studies describing any putative variation of the keratoconjunctivitis in relation to environmental influences such as pollen or occupational factors. Neither has there been any experimental evidence presented of the allergen response in this disorder. This is thus the first conjunctival pollen provocation study performed in AKC patients and the results should stimulate further trials.

A single high-dose challenge was conducted and revealed a typical IgE-mediated allergic reaction with temporary, but significant vasodilatation and chemosis of the conjunctiva along with itching. The result is certainly not unexpected, but in a chronic inflammatory condition like AKC, an acute phase reaction could

hypothetically be down-regulated. In contrast, the provocation evoked a response in the AKC patients comparable to that of the SAC group. The initial changes were normalized within a few hours and 8 h after provocation, the patients were free from symptoms. No changes were detectable in the cytokine concentrations at 8 h but, interestingly, 48 h after the provocation, a pattern with higher cytokine concentrations in the provoked eyes was proven in the AKC patients. This was demonstrated for IL-6 and IFN- γ vs. baseline and vs. the unprovoked eye and for IL-10 vs. the unprovoked eye. Much to our surprise, IL-5 and TNF- α remained undetectable in most samples both before and after provocation. TNF- α , being an acute phase cytokine, could possibly have been found in higher concentrations just after the challenge, but tear sampling was not performed until 8 h later. Our group has previously experienced varying results in IL-5 analyses with low levels in AKC patients in an eyelid treatment study [12], but more often, IL-5 has been reported as typically elevated in AKC patients [2, 5, 13], and an increase at 8 and possibly 48 h after provocation could have been expected parallel to an influx of eosinophils [14, 15]. Possibly, repeated challenge or an even higher allergen dose could have induced more pronounced changes [14, 16], but in a previous study in SAC patients, we found a single high-dose provocation suitable for a sufficient response [17] and this was here proven safe also for AKC patients.

An influence of both Th2 and Th1 cells and cytokines in AKC has earlier been demonstrated by Metz *et al.* and Calder *et al.* [6, 18] and has been confirmed in later studies [2, 4, 5, 13], but the relationship with disease activity is still unclear. Hingorani *et al.* found a reduction in T cell cytokine expression, especially in IL-2 and IFN- γ and also in IL-3 and IL-5 following treatment with topical Cyclosporine A in AKC patients underscoring the importance of both Th1 and Th2 cells [19], and our group found trends of reduction in IL-4 and IL-8 after eyelid treatment with Tacrolimus ointment [12]. Except for these treatment studies, there are no longitudinal studies demonstrating cytokine levels in relation to severity of the keratoconjunctivitis in AKC. Cross-sectional studies have provided varying results. Leonardi *et al.* showed a correlation between IFN- γ levels in tear fluid and corneal involvement [4]. We previously found associations between conjunctival signs and the levels of IFN- γ , TNF- α , IL-2, IL-4 and IL-10, but not for IL-5 [2], whereas Uhico *et al.* suggested IL-5 to be related to disease severity in AKC and not IFN- γ [13].

In atopic diseases such as AD, asthma and rhinitis, provocation studies with cytokine analyses have commonly been performed. Thepen *et al.* provoked AD patients with atopy patch test to airborne allergen and demonstrated an early-phase response with IL-4 producing T cells followed by late-phase peaking at 48 h

after provocation with a predominance of IFN- γ positive cells [20]. A late-phase reaction after provocation was confirmed in our study with an increase in IFN- γ in tear fluid and also an increase in IL-6, and a tendency towards an increase in IL-10 was seen. Regarding IL-10, divergent results have been presented in provocation studies of allergic asthma. Borish *et al.* reported reduced IL-10 levels in bronchoalveolar lavage fluid from asthmatics compared with controls both at baseline and after provocation [21], but several studies have demonstrated an increase after provocation [22, 23]. In rhinitis, similar conflicting data are shown [24, 25]. Our results verify that IL-10 is present in tears both in chronic allergy, seasonal allergy and in healthy controls, but an increase after provocation was shown after 48 h only in the AKC group. Moreover, the suggested anti-inflammatory IL-10 and pro-inflammatory IFN- γ correlated positively at all time-points in the AKC patients, which has previously not been demonstrated. In a cross-sectional study, Leonardi *et al.* demonstrated correlations between several cytokines in AKC, SAC and VKC, and correlations between IFN- γ and the Th2 cytokines IL-4 and IL-13, but not IL-10 were reported in the AKC group [5]. Repeated analyses as in the present study could possibly better reflect the intricate balance between these mediators.

IL-6 has also been found in higher concentrations after provocation in asthma [23] and we observed increased levels both in AKC and in SAC patients 48 h after challenge. The function of IL-6 in AKC is not known. In VKC, the concentration of soluble IL-6 receptor has been shown to correlate with disease activity, but this was not shown for AKC [26]. Incidentally, the IL-6 concentrations differed significantly at baseline between eyes in AKC patients in the present study with the eyes not to be challenged exhibiting higher values. This unexplained difference did not affect the outcome as the increased concentrations at 48 h after provocation was significant both vs. baseline and vs. the contralateral eyes. Together with IL-4, IL-13 is important in allergic reactions both by stimulating IgE production and by inducing a Th2-type response. Asthma studies have shown increased IL-13 secretion after provocation [27], and higher levels in AKC patients than in healthy controls have been documented [4], but this could not be observed in our study.

Considering baseline levels, all four detectable cytokines were found in AKC, and also in SAC and controls. A tendency towards higher values was seen in the AKC group, but due to low numbers in each group and some degree of baseline variation, a significant difference was proven only when analysing pooled data from both the left and the right eyes (data not shown). The study was not primarily designed to make comparisons between AKC and SAC and control subjects, respectively, but to

investigate the allergic response within the AKC group. We still had expected higher cytokine levels in the AKC patients than in SAC and control subjects, but the inclusion criterion for study participation – subjects being able to withhold maintenance therapy – favoured the selection of a cohort of patients with low-grade inflammation. Similar results with no difference in IFN- γ concentrations between AKC and CT and even higher levels of IL-10 in CT than in AKC patients have been published [13, 28]. In summary, the present results in part confirm, but in part also contradict, the published literature on cytokines in AKC and in all probability, this is due to differences in the inflammatory state of the patients examined and variability regarding sampling strategies and methods of analysis. Recent findings demonstrate that most cytokines are present also in tears from healthy subjects [29, 30], and that standardized methods for collection and evaluation are warranted.

We conclude that conjunctival challenge with a single allergen dose in AKC patients induces a significant,

but temporary conjunctival reaction accompanied by subclinical alterations in the cytokine levels 48 h later. Allergen exposure could be one factor of importance in exacerbating AKC responses. Larger studies comparing AKC patients with seasonal or perennial allergic conjunctivitis patients after conjunctival provocation might give further insights into the mechanisms of inflammation in AKC.

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The authors have no conflict of interest to declare.

References

- 1 Tuft SJ, Kemeny DM, Dart JK, Buckley RJ. Clinical features of atopic keratoconjunctivitis. *Ophthalmology* 1991; 98:150–8.
- 2 Nivenius E, Montan PG, Chryssanthou E, Jung K, van Hage-Hamsten M, van der Ploeg I. No apparent association between periorcular and ocular microcolonization and the degree of inflammation in patients with atopic keratoconjunctivitis. *Clin Exp Allergy* 2004; 34:725–30.
- 3 Garrity JA, Liesegang TJ. Ocular complications of atopic dermatitis. *Can J Ophthalmol* 1984; 19:21–4.
- 4 Leonardi A, Fregona IA, Plebani M, Secchi AG, Calder VL. Th1- and Th2-type cytokines in chronic ocular allergy. *Graefes Arch Clin Exp Ophthalmol* 2006; 244:1240–5.
- 5 Leonardi A, Curnow SJ, Zhan H, Calder VL. Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures. *Clin Exp Allergy* 2006; 36:777–84.
- 6 Metz DP, Hingorani M, Calder VL, Buckley RJ, Lightman SL. T-cell cytokines in chronic allergic eye disease. *J Allergy Clin Immunol* 1997; 100:817–24.
- 7 Möller C, Björkstén B, Nilsson G, Dreborg S. The precision of the conjunctival provocation test. *Allergy* 1984; 39:37–41.
- 8 Abelson MB, Chambers WA, Smith LM. Conjunctival allergen challenge. A clinical approach to studying allergic conjunctivitis. *Arch Ophthalmol* 1990; 108:84–8.
- 9 Abelson M, Howes J, George M. The conjunctival provocation test model of ocular allergy: utility for assessment of an ocular corticosteroid, loteprednol etabonate. *J Ocul Pharmacol Ther* 1998; 14:533–42.
- 10 Leonardi A, Busca F, Motterle L *et al.* Case series of 406 vernal keratoconjunctivitis patients: a demographic and epidemiological study. *Acta Ophthalmol Scand* 2006; 84:406–10.
- 11 Bonini S, Bonini S, Schiavone M, Centofanti M, Allansmith MR, Bucci MG. Conjunctival hyperresponsiveness to ocular histamine challenge in patients with vernal conjunctivitis. *J Allergy Clin Immunol* 1992; 89:103–7.
- 12 Nivenius E, van der Ploeg I, Jung K, Chryssanthou E, van Hage M, Montan PG. Tacrolimus ointment vs steroid ointment for eyelid dermatitis in patients with atopic keratoconjunctivitis. *Eye (Lond)* 2007; 21:968–75.
- 13 Uchio E, Ono SY, Ikezawa Z, Ohno S. Tear levels of interferon-gamma, interleukin (IL) -2, IL-4 and IL-5 in patients with vernal keratoconjunctivitis, atopic keratoconjunctivitis and allergic conjunctivitis. *Clin Exp Allergy* 2000; 30:103–9.
- 14 Bonini S, Bonini S, Bucci MG *et al.* Allergen dose response and late symptoms in a human model of ocular allergy. *J Allergy Clin Immunol* 1990; 86:869–76.
- 15 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; 106:948–54.
- 16 Kanek R, Matsu T, Iwatsuki K, Motok Y, Oyama N, Kaneko F. Biphasic cytokine expression by T cell clones from patients with atopic dermatitis with different incubation periods and strengths of stimuli. *Fukushima J Med Sci* 2001; 47:51–62.
- 17 Montan PG, van Hage-Hamsten M, Zetterström O. Sustained eosinophil cationic protein release into tears after a single high-dose conjunctival allergen challenge. *Clin Exp Allergy* 1996; 26:1125–30.
- 18 Calder VL, Jolly G, Hingorani M *et al.* Cytokine production and mRNA expression by conjunctival T-cell lines in chronic allergic eye disease. *Clin Exp Allergy* 1999; 29:1214–22.
- 19 Hingorani M, Calder VL, Buckley RJ, Lightman S. The immunomodulatory effect of topical cyclosporin A in atopic keratoconjunctivitis. *Invest Ophthalmol Vis Sci* 1999; 40:392–9.

- 20 Thepen T, Langeveld-Wildschut EG, Bihari IC *et al*. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial TH2 response to a TH1 response in situ: an immunocytochemical study. *J Allergy Clin Immunol* 1996; **97**:828–37.
- 21 Borish L, Aarons A, Rumbyrt J, Cvieta P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. *J Allergy Clin Immunol* 1996; **97**:1288–96.
- 22 Colavita AM, Hastie AT, Musani AI *et al*. Kinetics of IL-10 production after segmental antigen challenge of atopic asthmatic subjects. *J Allergy Clin Immunol* 2000; **106**:880–6.
- 23 Thunberg S, Gafvelin G, Nord M *et al*. Allergen provocation increases TH2-cytokines and FOXP3 expression in the asthmatic lung. *Allergy* 2010; **65**:311–8.
- 24 Muller B, de Groot EJ, Kortekaas IJ, Fokkens WJ, van Drunen CM. Nasal endothelial interleukin-10 expression is negatively correlated with nasal symptoms after allergen provocation. *Allergy* 2009; **64**:738–45.
- 25 Bensch GW, Nelson HS, Borish LC. Evaluation of cytokines in nasal secretions after nasal antigen challenge: lack of influence of antihistamines. *Ann Allergy Asthma Immunol* 2002; **88**:457–62.
- 26 Shoji J, Kawaguchi A, Gotoh A, Inada N, Sawa M. Concentration of soluble interleukin-6 receptors in tears of allergic conjunctival disease patients. *Jpn J Ophthalmol* 2007; **51**:332–7.
- 27 Huang SK, Xiao HQ, Kleine-Tebbe J *et al*. IL-13 expression at the sites of allergen challenge in patients with asthma. *J Immunol* 1995; **155**:2688–94.
- 28 Wakamatsu TH, Dogru M, Ayako I *et al*. Evaluation of lipid oxidative stress status and inflammation in atopic ocular surface disease. *Mol Vis* 2010; **16**:2465–75.
- 29 Carreño E, Enríquez-de-Salamanca A, Tesón M *et al*. Cytokine and chemokine levels in tears from healthy subjects. *Acta Ophthalmol* 2010; **88**:250–8.
- 30 Uchino E, Sonoda S, Nakao K, Sakamoto T. Alteration of tear cytokine balance by eye closure: analysis by multicytokine assay. *Graefes Arch Clin Exp Ophthalmol* 2006; **244**:747–9.