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Multiple cytokines in human tear specimens in seasonal and chronic allergic eye disease and in conjunctival fibroblast cultures

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Summary

Background Several cytokines are involved in the recruitment and activation of inflammatory cells in ocular allergic diseases. The purpose of the study was to assay multiple cytokines and chemokines in tears, to compare subgroups of allergic conjunctivitis (AC) with controls, and in culture supernatants to determine whether conjunctival fibroblasts produce some of these cytokines.

Methods Fifty to one hundred microlitre tears were obtained from patients with active seasonal allergic conjunctivitis (SAC; $n = 12$), vernal keratoconjunctivitis (VKC; $n = 18$), atopic keratoconjunctivitis (AKC; $n = 6$) and non-atopic controls ($n = 14$). Primary conjunctival fibroblasts grown *in vitro* were stimulated with IL-4, IL-13 or TNF- α for 24 h. Cell-free tear and culture supernatants were assayed for IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IFN- γ , TNF- α , eotaxin, MCP-1 and RANTES using multiplex bead analysis. Induction of chemokine gene expression was determined by PCR.

Results IL-1 β , IL-2, IL-5, IL-6, IL-12, IL-13, MCP-1 were increased in all tears groups compared with controls, with highly significant correlations between many of these molecules. In addition IL-4, IFN- γ , and IL-10 were elevated in SAC and VKC, while eotaxin and TNF- α were only increased in VKC. IL-6, IL-8, MCP-1, RANTES and eotaxin were detected from fibroblasts cultures, and were all up-regulated by TNF- α . By PCR, fibroblasts expressed MCP-1 transcripts constitutively, whereas IP-10 and Mig were up-regulated by TNF- α .

Conclusions Differential cytokine levels support tears as a useful indicator of immune mechanisms occurring during AC. The striking similarities in chemokine profiles between tears and fibroblasts suggest these cells as likely sources of chemokines in tears.

Keywords allergic conjunctivitis, chemokines, cytokines, tears

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Introduction

Allergic conjunctivitis (AC) describes a group of conditions ranging in severity from mild to severe. Seasonal allergic conjunctivitis (SAC) is the most frequently occurring ocular allergy, which is self-limiting and associated with environmental allergen exposure, involving predominantly mast cell degranulation within conjunctival

tissues. In contrast, vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) are rare but are severe diseases, usually recurrent, bilateral and chronic. VKC affects children and young adults, whereas AKC is typically associated with atopic dermatitis in adults. Both diseases can severely compromise the cornea, with ulcers and scarring ultimately leading to visual impairment. In both diseases, immunopathogenic mechanisms involve a combination of IgE- and T cell-mediated responses [1–4]. A massive inflammatory cell infiltrate has been widely described in cytological and immunohistochemical studies, with T cells producing pro-inflammatory cytokines such as IL-4, IL-5 and IL-13. In particular, increased

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numbers of activated eosinophils, which release pro-inflammatory and epitheliotoxic mediators [5, 6], are found in tears and tissues of all allergic eye diseases [7].

Several chemokines may be involved in the recruitment and activation of inflammatory cells in AC tissues. However, most studies have reported tissue expression of chemokines and chemokine receptors without quantitative analyses [8, 9]. For CXC chemokines, IL-8 (CXCL8) chemoattracts neutrophils, while IFN- γ -inducible protein of 10 kDa (IP-10; CXCL10) and monokine induced by IFN- γ (Mig; CXCL9), specifically chemoattract-activated T cells by signalling through the chemokine receptor CXCR3. MCP-1 (CCL2) and eotaxin (CCL11) are both members of the CC chemokine family, with MCP-1 a chemoattractant for monocytes and T cells, and eotaxin preferentially chemoattracting eosinophils by acting specifically on CCR3, which is highly expressed on eosinophils [10].

By generating T cell lines from conjunctival biopsies of allergic patients, specific T cell cytokine profiles have been found to predominate, with a Th2 cytokine profile in VKC, and a shift towards a Th1 cytokine profile in AKC [3]. Increased expression of IL-4 and IL-13 has also been shown in SAC [11], where mast cells and Th2-type cytokines are thought to orchestrate the late-phase reaction. Nevertheless, other cell types and multiple cytokines are likely to be involved in the complex pathogenesis of seasonal and chronic allergic eye diseases.

It has previously been suggested that tears can be used as a representation of what is occurring *in vivo* at the ocular surface. Although only small numbers of T cells are present in tears, intracellular cytokine staining has been used to characterize VKC tears-derived T cell populations [2], which were found to be IL-4-positive, similar to those from VKC conjunctival biopsies [1]. Tear samples are relatively easy to collect, avoiding the need for biopsying, and provide valuable information on differential tear cytology and mediators at the ocular surface. The critical limitation of these measurements is the tear volume of the sample, which ideally should be collected without stimulating the tear reflex, to avoid diluting the mediators.

A quantitative method for simultaneously assaying six cytokines in tears has been described in allergic and non-allergic patients, showing significant differences in the ratios of pro-inflammatory cytokines between the two groups [12]. Tear cytokine levels have been reported as low or undetectable in SAC compared with VKC and AKC [6], yet relatively few cytokines have been measured simultaneously in the same tear sample. In the present study, 10 pro-inflammatory cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFN- γ and TNF- α) and four chemokines (IL-8, RANTES, eotaxin and MCP-1) were measured in tears by multiplex bead analysis, comparing different subgroups of AC with controls. As the source of the tear cytokines and chemokines is unknown, primary

cultures of conjunctival fibroblasts were also investigated for their ability to secrete cytokines and chemokines in response to treatment with IL-4, IL-13 or TNF- α , all of which are present within conjunctival tissues during inflammation. Finally, in addition to MCP-1, chemokines that are involved in attracting T cells (IP-10, MIG) but that are not available for multiplex analysis, were studied by PCR in conjunctival cells to determine their potential role in recruiting T cells to VKC and AKC conjunctival tissues.

Materials and methods

Patients

Tear samples were collected from patients with active and non-treated allergic disease who presented to the Ophthalmology Department of Padua University as new referrals or with a reactivation of the disease: 18 with VKC (12 ± 5 years [mean $\pm$ SD]; range 4–27; 14 males), 12 with SAC (25 ± 9 years; range 12–40; eight males) and six with AKC (26 ± 7 years; range 15–35; all males). For SAC patients, tears were taken during the pollen season. Tear samples were also obtained from 14 normal volunteers (31 ± 7 ; range 12–37; 10 males; four females). Exclusion criteria for the control subjects were: history of allergic disease, any sign or symptom of external eye disease and any use of drugs for at least 1 month.

Written informed consent was obtained from all subjects or, in the case of minors, from their parents after explanation of the nature and the possible consequences of the study. The research followed the Tenets of the Declaration of Helsinki. Institutional Review Board approval was obtained. Diagnosis of each inflammatory disease was established according to the patient's history, the clinical signs and symptoms, skin test results, and the determination of serum total and specific IgE. Exclusion criteria for SAC, VKC and AKC patients were: use of any topical drugs for at least 5 days and systemic anti-allergic treatment for at least 2 weeks.

In all patients, a subjective clinical score (0–10) was given considering the overall severity of the disease (0 = no symptoms and no active signs; 10 = very severe symptoms and signs with corneal ulcer) at the time of sample collection. Additionally, an individual score for corneal involvement was given: 0 = clear cornea, 1 = local punctate keratitis, 2 = diffuse punctate keratitis; 3 = focal confluent de-epithelization; 4 = corneal shield ulcer. All patients and normal volunteers were free of medication for at least 3 days before sample collection.

Tear collection

Using a capillary micro-pipette, tears were gently collected from the external canthus (avoiding the tear reflex as much as possible) and placed in Eppendorf tubes. Fifty

to one hundred microlitres of tears were collected from each subject. Tears were collected from the most affected eye or pooled from both eyes if the condition was clinically symmetrical. Two microlitres aliquots of non-centrifuged tear samples were used for cytology, which was evaluated using pre-coloured slides (Testsimplets[®], Roche, Mannheim, Germany), counting the eosinophils, neutrophils and lymphocytes present in five microscopic fields of 0.15 mm². The remaining tear fluid was centrifuged at 160 g for 10 min. Supernatants were then stored at -80 °C for subsequent determination of cytokines and chemokines.

Conjunctival fibroblasts

Fibroblast cultures were obtained from upper tarsal conjunctival biopsies of two normal subjects who underwent cataract surgery. Anesthesia was induced by topical 2% lidocaine. Briefly, biopsies were washed, cut in small pieces, seeded in culture wells (NUNC, Roskilde, Denmark) containing 100 µL of Ham's F12 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Sigma), L-glutamine (2 mM) and antibiotics (penicillin 100 U/mL, streptomycin 100 mg/mL; Sigma). Samples were incubated at 37 °C in 5% CO₂ in a humidified air atmosphere. Fibroblasts were subcultured with 0.05% trypsin and replated into 24-well plates (>95% vitality). Cells were characterized morphologically and stained positively with vimentin and negatively with cytokeratins. Third- to fifth-passage fibroblasts were used for experiments.

To evaluate the effects of IL-4, IL-13 and TNF- on cytokine and chemokine production, cells were seeded into 24-well plates in the presence of the appropriate medium and 10% FCS. At confluence, the medium in each well was replaced with 500 µL of serum-free medium. After 24 h, the medium was replaced with fresh medium supplemented with 10 ng/mL of human recombinant IL-4, IL-13 or TNF-α (Peprotech, London, UK). After 24 h, the medium was removed, centrifuged and stored at -80 °C. Three culture wells were used for each treatment and experiments repeated twice.

Multiplex bead analysis

Samples were diluted 1 : 1 and analysed using multiplex bead analysis, which utilizes microspheres as the solid support for immunoassays and allows the analysis of all molecules from each sample. IL-1β, -2, -4, -5, -6, -8, -10, -12, -13, TNF-α, IFN-γ, eotaxin, MCP-1 and RANTES were measured according to the manufacturer's instructions (Upstate Biotechnology UK, Buckingham, UK). Briefly, samples were incubated with antibody-coated capture beads for 2 h at 20 °C. Washed beads were further incubated with biotin-labelled anti-human cytokine antibodies for 1 h followed by streptavidin-phycoerythrin for

30 min. Samples were analysed using a Luminex 100[™] (Luminex, Austin, TX, USA) with Starstation software (Applied Cytometry Systems, Sheffield, UK). Standard curves of known concentrations of recombinant human cytokines were used to convert fluorescence units to cytokine concentration (pg/mL). Minimum detection levels for each cytokine were as follows: 20 (IL-2), 10 (IL-1β, IL-8, IL-12, IL-13, TNFα, MCP-1, RANTES), five (IL-4, IFN-γ), two (IL-5, IL-10, eotaxin), one (IL-6) pg/mL.

Polymerase chain reaction

Cultured cells were pelleted at 48 h and total RNA extracted using an RNeasy extraction kit according to the manufacturers' instructions (Qiagen Ltd., Crawley, UK), as described previously [3]. Samples were resuspended in 100 µL of buffer containing 90 mM Tris-HCl, pH7.5, 10 mM MgCl₂, 50 mM NaCl and treated with 10 U RNA-free DNAase for 15 min at 37 °C. RNA was extracted twice with phenol : chloroform (1 : 1), once with chloroform, precipitated in ethanol and resuspended in diethylpyrocarbonate-treated distilled water. RNA was quantitated by UV absorbance at 260 nm. cDNA was prepared from 1 to 2 µg oligo-d(T) primed RNA by standard methods using AMV reverse transcriptase (Invitrogen, Paisley, UK). Two microlitres samples of single strand cDNA were cycled between 30 and 40 times with 1 U Taq DNA polymerase (Stratagene, Cambridge, UK), 200 µM dNTPs and 0.2 µM of each primer in buffer containing 5 mM MgCl₂. Human specific oligonucleotide primers were used to amplify: β-actin (3'-GAG CACAGAGCCT-CGCCTTTC-5'), IP-10 (3'-AGAGGAAC-CTCCAGTCTCAGC-5'), Mig (3'-TTA-AACAATTGCCCCA AGC5'), MCP-1 (3'-AGTCCAACCAAGGTTTGTGC-5') transcripts. PCR was conducted in a Hybaid Omnigene thermal cycler (Hybaid, Ashford, UK). The pre-optimized PCR conditions were denaturation 5 min at 94 °C, followed by 30–40 cycles of annealing 30 s at 55 °C and extension 30 s at 72 °C for β-actin, with an extra 7 min extension at 72 °C for IP-10, Mig and MCP-1. The identity of the amplified product was confirmed by agarose gel electrophoresis, followed by ethidium bromide staining under UV illumination. Only samples demonstrating expression of β-actin transcripts at 620 bp were subsequently screened for cytokine expression. All amplicons were of the size predicted from the cDNA sequence, with IP-10 at 355 bp, Mig at 380 bp and MCP-1 at 411 bp. PCR products from each amplification protocol were isolated and cycle-sequenced on an ABI PRISM automated DNA sequencer to confirm the identity of the amplification for each cytokine.

Statistical analysis

All of the cytokine and chemokine data sets were analysed for normal distribution using the Kolmogorov–Smirnov

normality test. As the majority of the data sets were not normally distributed, the non-parametric Kruskal–Wallis analysis with Dunn's multiple comparison post-test was used. Correlations were calculated using Spearman's correlation coefficient. The minimal level of confidence at which the results were judged significant was $P < 0.05$. Data are reported as mean $\pm$ standard deviation.

Results

Cytokine and chemokine analysis in tears

All 14 cytokines and chemokines were measurable in tear samples from allergic subjects. For the majority of controls, most cytokines were below the detection limit ($< 1\text{--}10\text{ pg/mL}$), with only a few samples showing detect-

able levels of IL-6, IL-13, MCP-1 and RANTES (Fig. 1). IL-8 was present in all control tears and was not significantly elevated in AC groups (Fig. 1). In comparison with controls, the levels of IL-1 β , IL-2, IL-5, IL-6, IL-12, IL-13, and MCP-1 were significantly elevated in tears from SAC, VKC and AKC; IL-4, IFN- γ and IL-10 were elevated in SAC and VKC (Table 1), and eotaxin and TNF- α were only increased in VKC. Comparisons among the groups found that tear levels of IL-5, RANTES and eotaxin were significantly higher in VKC compared with SAC. When VKC patients were subdivided into those with positive or negative serum specific IgE (atopic vs. non-atopic VKC), no significant differences were found in levels of cytokine or chemokine.

For SAC, VKC and AKC, correlations between cytokines in individual tears were investigated. In all three diseases,

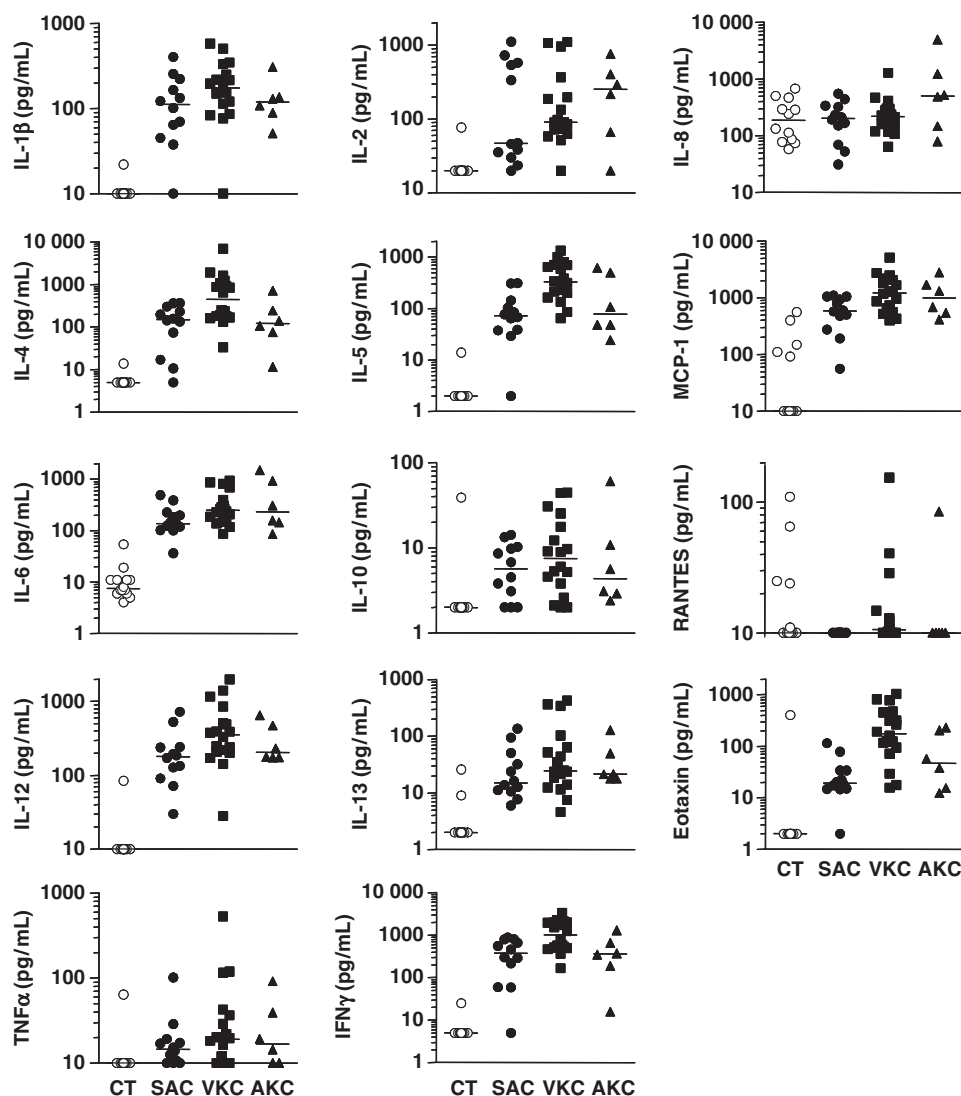


Fig. 1. Cytokine and chemokine tear levels in healthy control subjects (CT) and patients affected by seasonal allergic conjunctivitis (SAC), vernal keratoconjunctivitis (VKC), and atopic keratoconjunctivitis (AKC) measured by multiplex bead immunoassay. Bars represent the median value for each group. P values of cytokine levels comparisons are reported in Table 1.

Table 1. Comparison of cytokine and chemokine levels between control (CT), VKC, AKC and SAC tear samples

	IL-1 β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12	IL-13	MCP-1	RANTES	EOTAXIN	TNF α	IFN γ
VKC vs. CT	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	NS	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	NS	$P < 0.001$	$P < 0.01$	$P < 0.001$
SAC vs. CT	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.05$	$P < 0.01$	NS	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$	NS	NS	NS	$P < 0.01$
AKC vs. CT	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$	$P < 0.001$	NS	NS	$P < 0.01$	$P < 0.01$	$P < 0.01$	NS	NS	NS	NS
VKC vs. SAC	NS	NS	NS	$P < 0.05$	NS	NS	NS	NS	NS	NS	$P < 0.05$	$P < 0.05$	NS	NS

NS, not significant; VKC, vernal keratoconjunctivitis; AKC, atopic keratoconjunctivitis; SAC, seasonal allergic conjunctivitis.

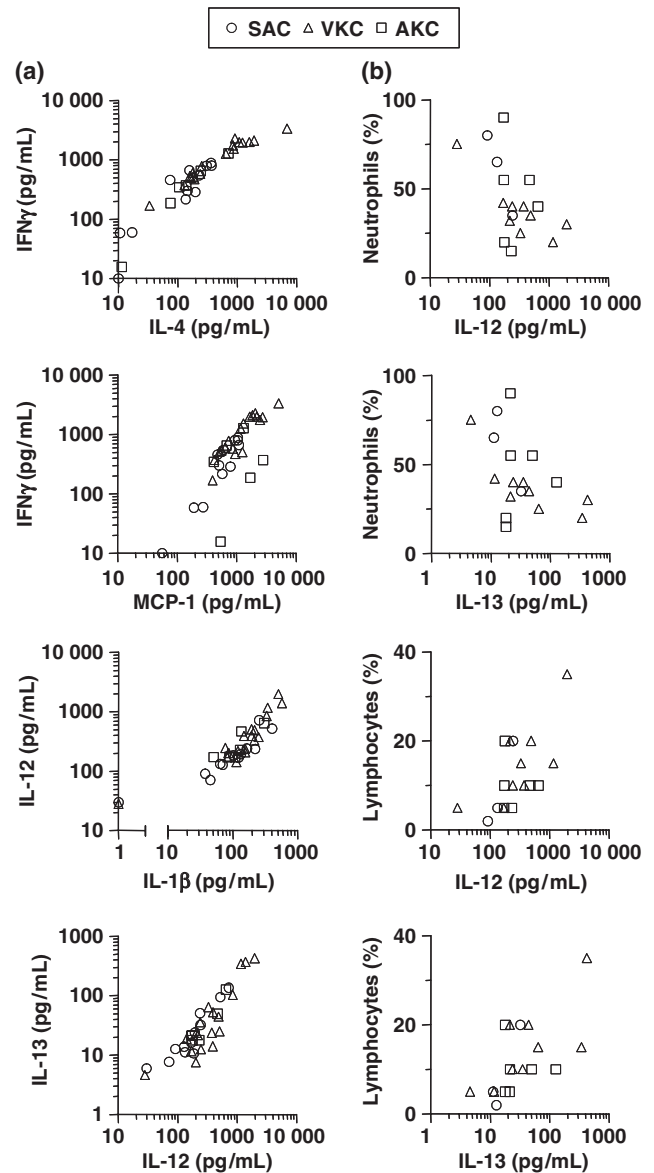


Fig. 2. Correlation between pairs of cytokines (a) and between cytokines and the percentage of lymphocytes or neutrophils (b) in the tear samples of patients affected by seasonal allergic conjunctivitis (SAC; $\circ$), vernal keratoconjunctivitis (VKC; Δ), atopic keratoconjunctivitis (AKC; squares).

IL-4 was strongly correlated with both IFN- γ (Fig. 2a) and IL-13. Numerous other significant correlations were obtained (data not shown). IL-1 β , IL-4, IL-12, IL-13, IFN- γ , eotaxin-1 and MCP-1 appeared to be closely associated. For each disease group, the correlations between cytokines were very similar, with the major differences between groups being the levels of cytokines.

At the time of tear sample, the mean severity score (0–10) was 3.5 ± 1.1 (range 1–5) in SAC, 6.5 ± 2.2 (range 4–10) in VKC and 5.5 ± 1.7 (range 4–9) in AKC. Significant correlations between clinical scores and tear IL-1 β ($r = 0.8$, $P < 0.01$), IL-4 ($r = 0.71$, $P < 0.01$), IL-5

($r=0.8$, $P < 0.01$), MCP-1 ($r=0.8$, $P < 0.01$), eotaxin-1 ($r=0.8$, $P < 0.01$) and IFN- γ ($r=0.86$, $P < 0.001$) were observed only in SAC. No significant relationship between overall clinical status and any cytokine level was identified in VKC or AKC. The cornea was involved in 13 of 18 VKC patients (mean score 1.6 ± 1.3), in six of six AKC patients (1.5 ± 1.2), while it was absent in SAC. Corneal disease activity score correlated significantly with IL-5 in VKC ($r=0.43$, $P < 0.05$) but not in AKC.

Tear cytology identified the percent inflammatory cells as follows: $49 \pm 15\%$ eosinophils, $37 \pm 15\%$ neutrophils, and $15 \pm 9\%$ lymphocytes in VKC; $44 \pm 25\%$ eosinophils, $45 \pm 27\%$ neutrophils and $10 \pm 5\%$ lymphocytes in AKC; $20 \pm 14\%$ eosinophils, $70 \pm 18\%$ neutrophils and $9 \pm 7\%$ lymphocytes in SAC. For the VKC group, sufficient data was available to identify relationships between cellular infiltrate and cytokine in the same tear sample. Positive correlations were found between the percentage of lymphocytes and both IL-12 ($r=0.71$, $P < 0.05$) and IL-13 ($r=0.71$, $P < 0.05$), and negative correlations were found between neutrophils and IL-12 ($r=-0.72$, $P < 0.05$) and IL-13 ($r=-0.85$, $P < 0.01$) (Fig. 2b). Although the majority of cells were eosinophils, their percentage did not correlate with tear levels of cytokines or chemokines involved in eosinophil migration and/or activation.

Quantitation of cytokines in conjunctival fibroblast cultures

IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IFN- γ and TNF- α were not detected either at baseline or after 24 h exposure to cytokines, with the exception of IL-4, IL-13 and TNF- α , which were detected in those cultures to which the respective cytokines were added.

In untreated fibroblast cultures, IL-6, IL-8, MCP-1 and RANTES were detected and were significantly increased by TNF- α ($P < 0.01$). In contrast, eotaxin-1 was not

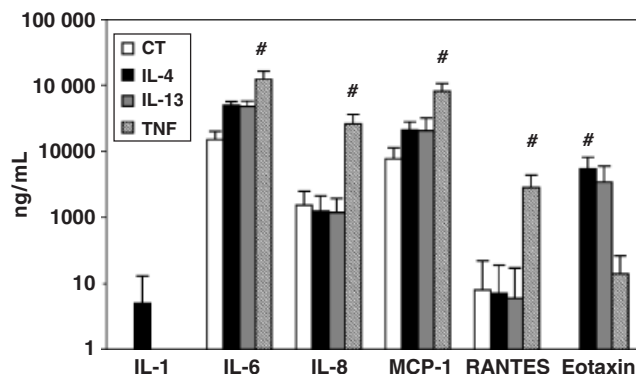


Fig. 3. Cytokine levels in 24 h culture supernatants from cultures of primary conjunctival fibroblasts, comparing non-atopic controls (CT) (open bar) with IL-4-treated (solid), IL-13-treated (grey) and TNF- α -treated (hatched) cells. * $P < 0.05$; # $P < 0.01$.

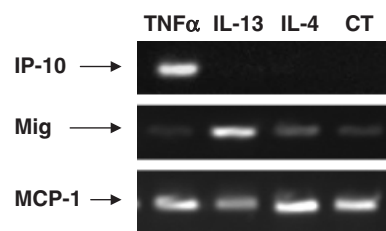


Fig. 4. Gel electrophoresis of RT-PCR products, demonstrating relative expression of IP-10, Mig and MCP-1 transcripts by primary conjunctival fibroblasts.

produced by untreated cells but was induced by IL-4, IL-13 and, to a lesser extent, by TNF- α (Fig. 3). IL-1 β was only detected in low levels in response to IL-4.

The effects of cytokines on IP-10, MCP-1 and Mig mRNA expression by fibroblasts

By PCR, unstimulated fibroblasts did not express IP-10 transcripts, but IP-10 mRNA expression in fibroblasts was induced in response to TNF- α but not IL-4 or IL-13 (Fig. 4). Similarly Mig expression was detected very weakly in untreated fibroblasts; and was unchanged in response to either IL-4 or TNF- α , but IL-13 significantly up-regulated Mig expression (Fig. 4). MCP-1 was constitutively expressed, and no changes in intensity of transcripts were detected in response to cytokines.

Discussion

Cytokines have previously been measured in tears of active allergic patients and normal subjects using ELISA [6, 13], but limited sample volumes have restricted analyses to only one or two cytokines. A recent study of human tears using membrane-bound antibody technology reported detection of 40 of 79 bioactive proteins, which was highly sensitive but not quantitative [14]. The multiplex bead immunoassay technology quantifies, in small volumes, a wide range of cytokines and chemokines, providing further information as to relative levels and correlations between cytokines within individual samples [12]. This technology has been used to study the diurnal rhythm of tear cytokines and the effects of eye closure and tear reflex on their balance in normal volunteers [15–17].

Using multiplex bead analysis of tears in active SAC, VKC and AKC, several cytokines and chemokines were significantly increased when compared with controls, with many Th2, Th1 and pro-inflammatory cytokines elevated, and IL-1 β , IL-6 and TNF- α were increased in active VKC, confirming previous reports [13, 18]. Even in SAC, a self-limiting disorder resulting from specific mast cell activation, there were significant increases in IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IFN- γ and MCP-1 in comparison with the controls, suggesting other cells apart

from mast cells are involved. In fact, apart from SAC having lower mean levels, there appeared to be little difference between SAC and VKC or AKC in terms of cytokine profile (Fig. 1).

There was a positive correlation between the percentages of tears-containing lymphocytes and IL-12 and -13 levels, whereas no association was found with eosinophils, which was surprising as it is well known that eosinophil recruitment and activation are responsible for conjunctival inflammation and corneal damage in ocular allergic diseases [7, 19]. This anomaly may be due to the cytology data being presented as percentage of cells, rather than the percentage or the actual number of activated eosinophils, which might be more physiologically relevant [19]. In VKC, a significant increase in eotaxin-1 was demonstrated, confirming a previous report of high levels of eotaxin-1 and -2 in active VKC tears and their correlation with eosinophil count and disease severity [20].

Although SAC, VKC and AKC tears contained different levels of cytokines, there was no obvious Th1 or Th2 profile for any disease group, and there were even positive correlations between Th1 and Th2 cytokines (e.g. IL-4 and IFN- γ). A similar tear cytokine pattern profile was found in tears of AKC patients using a similar multiplex method [21]. These findings suggest that a wide range of cytokines is involved by complex interwoven interactions rather than distinct and parallel pathways and that antagonistic cytokines attempt to regulate each other. As many cytokines and chemokines are produced by inflammatory cells, and by stimulated stromal cells, such as epithelial cells and fibroblasts, it is likely that the latter acted as a critical source of many of the tears cytokines detected. In fact, tear cytokines correlated poorly with tear cytology, suggesting that the cytokines were not produced by cells present in the tears, but by resident conjunctival tissue cells.

Investigating the conjunctival fibroblasts revealed a striking similarity with the chemokine profiles detected in tear samples. Fibroblasts constitutively produced IL-6, IL-8, MCP-1 and RANTES. However, other ocular surface cells including epithelial cells, intraepithelial lymphocytes (CD8⁺), dendritic cells, and resident mast cells are potential sources of these cytokines. The demonstration in the present study of eotaxin-1 production by conjunctival fibroblasts in response to IL-4 strongly suggests that these cells produce this chemokine, which is detected in tears.

Several chemokines may be involved in the pathogenesis of ocular allergic diseases [8, 9]. Immunohistochemical staining demonstrated IL-8, MCP-1 and RANTES was localized to conjunctival tissues in biopsies from AKC and VKC [8, 22]. Furthermore, mediators involved in the allergic cascade have been shown to result in activation of conjunctival and corneal fibroblasts [23–26].

Chemokines may be one factor involved in driving a pre-disposed allergic patient toward a specific ocular manifestation, perhaps at the level of differential cell recruitment to the inflammatory site [27]. For this reason, the ability of conjunctival resident cells to express chemokine mRNA was investigated. The expression of the CXC chemokines (IP-10, Mig) by conjunctival fibroblasts in response to pro-inflammatory cytokines further supports a role for these cells in recruitment of T cells during chronic allergic eye disease.

In conclusion, multiplex bead assays offer several advantages for detecting cytokines in small sample volumes, as they are highly sensitive, and allow a quantitative analysis of a wide range of cytokines in a single sample. The three allergic conditions considered here have unique clinical features and prognoses, as well as a different response to anti-allergic drugs and immunosuppressive therapy. Results of this tears study reveal that these diseases differ predominantly in the quantity, rather than the quality, of cytokine present. The similar chemokine profiles observed in conjunctival fibroblasts and their cytokine-induced responses, support a role for these cells during conjunctival inflammation. Comparison of multiple cytokine production is a new tool that may be used for understanding the many cytokine pathways involved in allergic diseases and, ultimately, how these can be modulated pharmacologically in the search for new definitive anti-allergic treatments.

References

- 1 Maggi E, Biswas P, Del Prete G *et al.* Accumulation of Th-2-like helper T cells in the conjunctiva of patients with vernal conjunctivitis. *J Immunol* 1991; **146**:1169–74.
- 2 Leonardi A, DeFranchis G, Zancanaro F *et al.* Identification of local Th2 and Th0 lymphocytes in vernal conjunctivitis by cytokine flow cytometry. *Invest Ophthalmol Vis Sci* 1999; **40**: 3036–40.
- 3 Calder VL, Jolly G, Hingorani M *et al.* Cytokine production and mRNA expression by conjunctival T cell lines in chronic allergic eye diseases. *Clin Exp Allergy* 1999; **29**:1214–22.
- 4 Montan PG, Scheynius A, van der Ploeg I. Similar T helper Th2-like cytokine mRNA expression in vernal keratoconjunctivitis regardless of atopic constitution. *Allergy* 2002; **57**:436–41.
- 5 Trocme SD, Aldave AJ. The eye and the eosinophil. *Surv Ophthalmol* 1994; **39**:241–52.
- 6 Leonardi A, Borghesan F, DePaoli M, Plebani M, Secchi AG. Tear and serum soluble leukocyte activation markers in conjunctival allergic diseases. *Am J Ophthalmol* 2000; **129**:151–8.
- 7 Trocme SD, Hallberg CK, Gill KS, Gleich GJ, Tying SK, Brysk MM. Effects of eosinophil granule proteins on human corneal epithelial cell viability and morphology. *Invest Ophthalmol Vis Sci* 1997; **38**:593–9.
- 8 Abu El-Asrar AM, Struyf S, Al-Kharashi SA, Missotten L, Van Damme J, Geboes K. Chemokines in the limbal form of vernal keratoconjunctivitis. *Br J Ophthalmol* 2000; **84**:1360–6.

- 9 Abu El-Asrar AM, Struyf S, Al-Kharashi SA, Missotten L, Van Damme J, Geboes K. The T-lymphocyte chemoattractant Mig is highly expressed in vernal keratoconjunctivitis. *Am J Ophthalmol* 2003; 136:853–60.
- 10 Baggiolini M, Dewald B, Moser B. Human chemokines: an update. *Ann Rev Immunol* 1997; 15:675–705.
- 11 Fujishima H, Saito I, Takeuchi T, Shinozaki N, Tsubota K. Characterization of cytokine mRNA transcripts in conjunctival cells in patients with allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 1997; 38:1350–7.
- 12 Cook EB, Stahl JL, Lowe L et al. Simultaneous measurement of multiple cytokines in a single sample of human tears using microparticle-based flow cytometry. *J Immunol Methods* 2001; 254:109–18.
- 13 Leonardi A, Borghesan F, DePaoli M, Plebani M, Secchi AG. Procollagens and inflammatory cytokine concentrations in tarsal and limbal vernal keratoconjunctivitis. *Exp Eye Res* 1998; 67:105–12.
- 14 Sack RA, Conradi L, Krumholz D, Beaton A, Sathe S, Morris C. Membrane array characterization of 80 chemokines, cytokines, and growth factors in open- and closed-eye tears: angiogenin and other defense system constituents. *Invest Ophthalmol Vis Sci* 2005; 45:1228–38.
- 15 Uchino E, Sonoda S, Kinukawa N, Sakamoto T. Alteration pattern of tear cytokines during the course of a day: diurnal rhythm analyzed by multicytokine assay. *Cytokine* 2006; 33:36–40.
- 16 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* 2005; 11:1–3.
- 17 Sonoda S, Uchino E, Nakao K, Sakamoto T. Inflammatory cytokine of basal and reflex tears analysed by multicytokine assay. *Br J Ophthalmol* 2006; 90:120–2.
- 18 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.
- 19 Hingorani M, Calder V, Jolly G, Buckley RJ, Lightman SL. Eosinophil surface antigen expression and cytokine production vary in different ocular allergic diseases. *J Allergy Clin Immunol* 1998; 102:821–30.
- 20 Leonardi A, Jose PJ, Zhan H, Calder VL. Tear and mucus eotaxin-1 and eotaxin-2 in allergic keratoconjunctivitis. *Ophthalmology* 2003; 110:487–92.
- 21 Nivenius E, Montan PG, Chrysanthou E, Jung K, van Hage-Hamsten M, van der Ploeg I. No apparent association between periocular and ocular microcolonization and the degree of inflammation in patients with atopic keratoconjunctivitis. *Clin Exp Allergy* 2004; 34:725–30.
- 22 Hingorani M, Calder VL, Buckley RJ, Lightman SL. The role of conjunctival epithelial cells in chronic ocular allergic disease. *Exp Eye Res* 1998; 67:491–500.
- 23 Kumagai N, Fukuda K, Nishida T. Synergistic effect of TNF-alpha and IL-4 on the expression of thymus- and activation-regulated chemokine in human corneal fibroblasts. *Biochem Biophys Res Commun* 2000; 279:1–5.
- 24 Leonardi A, Radice M, Fregona IA, Plebani M, Abatangelo G, Secchi AG. Histamine effects on conjunctival fibroblasts from patients with vernal conjunctivitis. *Exp Eye Res* 1999; 68:739–46.
- 25 Fukuda K, Yamada N, Fujitsu Y, Kumagai N, Nishida T. Inhibition of eotaxin expression in human corneal fibroblasts by interferon-gamma. *Int Arch Allergy Immunol* 2002; 129:138–44.
- 26 Leonardi A, Cortivo R, Fregona I, Plebani M, Secchi AG, Abatangelo G. Effects of Th2 cytokines on expression of collagen, MMP-1, and TIMP-1 in conjunctival fibroblasts. *Invest Ophthalmol Vis Sci* 2003; 44:183–9.
- 27 Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. *Nat Rev Immunol* 2002; 2:982–7.