

Immunolocalization of cytokines to mast cells in normal and allergic conjunctiva

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Summary

Background Recently, the potential role of mast cells in allergic reactions has been extended by the discovery that these cells synthesize, store and secrete multifunctional cytokines. Seasonal allergic conjunctivitis is characterized as an immediate hypersensitivity reaction, in which allergen binds to specific IgE on mast cells, leading to release of pre-formed and newly synthesized inflammatory mediators.

Objective In this study we aimed to localize the cytokines IL-4, IL-5, IL-6, IL-8 and TNF α to conjunctival mast cells and to examine the relationship between mast cell-associated cytokines and allergic conjunctivitis.

Methods Immunohistochemistry was performed on serial sections of conjunctival biopsies from patients with seasonal allergic conjunctivitis, in and out of the hay fever season, as well as from non-allergic volunteers.

Results IL-4, IL-5, IL-6 and TNF α were localized to mast cells in normal and allergic conjunctiva. IL-8 was localized to mast cells in two patients with seasonal allergic conjunctivitis, one during and the other outside the pollen season. Using the monoclonal antibody 3H4, which identifies the secreted form of IL-4, biopsies from patients with active seasonal allergic conjunctivitis contained a significantly higher proportion of mast cells positive for IL-4, than those from out-of-season patients ($P = < 0.016$). There was no difference between the two groups in the number of mast cells immunostained by the antibody 4D9 which identifies the stored form of IL-4.

Conclusions These results suggest that conjunctival mast cells can store a range of multifunctional cytokines and release IL-4 during active disease, which may give them an important role in upregulating allergic inflammation in the conjunctiva.

Keywords: mast cell, cytokine, allergic conjunctivitis

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Introduction

There is considerable evidence that the mast cell is central to the pathogenesis of seasonal allergic conjunctivitis (SAC). This disease is accompanied by mast cell hyperplasia, by migration of mast cells into conjunctival epithelium [1], and by an increase in tear levels of the mast cell neutral protease tryptase [2]. Challenge of sensitized individuals with pollen extract reproduces the features of ocular allergy, and these

are associated with increased tear levels of the inflammatory mediators histamine, prostaglandin D₂ and leukotrienes C₄ and D₄ [3,4], all of which are synthesized and secreted by mast cells. Topical application of levocabastine, a highly specific H₁ anti-histamine, moderates symptoms in SAC [5] as well as after allergen challenge [6].

In addition to immediate changes which can be explained by the IgE-dependent release of mast cell products, a late-phase reaction may occur several hours after challenge with high doses of allergen. In this reaction persisting symptoms are associated with an increase in eosinophils and neutrophils in conjunctival scrapings and tears [7,8] and are not

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prevented by pre-treatment with levocabastine [9]. Similarly, in SAC eosinophils are found in conjunctival scrapings from some, but not all, symptomatic patients [10,11].

Following initial observations in rodents [12], mast cells from human skin or nasal mucosa have now been shown to store and secrete a range of multifunctional cytokines, including interleukin-4 (IL-4), IL-5, IL-6, [13,14], IL-8 [15], and tumour necrosis factor- α (TNF α) [16]. The individual properties of these cytokines suggest that the mast cell has the capacity, in addition to its accepted role as an effector cell, to upregulate the local allergic response and to organize the participation of other inflammatory cells.

IL-4 controls the isotype switch of B cells to IgE production [17] enhances mast cell arachidonate release following IgE-dependent activation [18] and causes naive CD4⁺T cells to acquire the TH₂ phenotype [19] with upregulation of the IL-4 gene cluster of cytokines encoded on chromosome 5 [20]. IL-4 is also required for the upregulation of vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin superfamily of adhesion molecules, involved in selective recruitment of eosinophils from the microvasculature [21]. Over-expression of IL-4 in transgenic mice leads to a marked increase in serum IgE and a severe inflammatory blepharitis which has a marked mast cell and eosinophil infiltration [22].

IL-5 is a powerful and selective stimulator of eosinophil differentiation, priming and chemotaxis [23]. IL-6 induces the differentiation of B cells into immunoglobulin-secreting plasma cells [24] potentiates IL-4-dependent IgE synthesis by B cells [25] and stimulates the proliferation of peripheral T cells [26]. IL-8 was originally identified as a potent chemoattractant and activator of neutrophils [27] but is now also recognized as a potent chemoattractant for cytokine-primed eosinophils [28]. TNF α induces VCAM-1 and intercellular adhesion molecule-1 (ICAM-1) [29], which are important in the selective recruitment of eosinophils, and also through the induction of the adhesion molecule E-selectin [16] contributes to the recruitment of neutrophils in IgE-dependent cutaneous late phase reactions [30].

Stimulation of mast cells via the IgE receptor has been shown to promote the synthesis of IL-4 [12], IL-5 [31], IL-8 [15], and TNF α [32]. Cross-linkage of Fc ϵ RI in human lung mast cells induced IL-4 production, maximal at 2 h, and led to an increase in IL-5 and TNF α mRNA signals within 2 h, but had no effect on IL-6 and IL-8 mRNA expression [31]. There was also constitutive expression of mRNA for IL-5, IL-6, IL-8 and TNF α .

Because of the potential importance of cytokines in mediating inflammation in allergic disease, in the present study we have investigated the localization of specific cytokines to conjunctival mast cells, and have examined the relationship between mast cell cytokine stores and disease activity in allergic conjunctivitis.

Table 1. Individual mast cell counts/mm², by group (SAC, seasonal allergic conjunctivitis in season; OOS, out of season). *P*-values: normal vs SAC=0.34, normal vs OOS=0.08, SAC vs OOS=0.92

	Normals	SAC	OOS
	6.0	16.5	10.9
	8.5	17.3	17.6
	11.6	23.2	18.4
	15.2	23.2	21.9
	15.2	24.7	26.9
	16.4	27.2	31.7
	22.2	33.9	35.1
	28.7	52.5	46.4
	30.7		
Median	15.2	23.9	24.4

Materials and methods

The study was approved by the Joint Ethical Committee of the University of Southampton and South-west Hampshire Health Authority, and all subjects gave their written informed consent.

Subjects

Conjunctival biopsies were taken from (a) nine normal subjects, immediately prior to cataract surgery (two male, seven female, mean age 67.5 years), (b) eight subjects during the pollen season with active symptoms of SAC (five male, three female, mean age of 50.1 years) and (c) eight subjects outside the pollen season with a history of SAC (six male, two female, mean age 40 years).

All subjects were assessed using a slit lamp biomicroscope, and scored for features of allergic conjunctivitis using the system of Abelson [33]. SAC was diagnosed on the basis of a positive skin-prick test to mixed grass pollen extract (Soluprick 299, ALK Laboratories, Denmark) and either typical clinical features of SAC (group b) or a typical history of previous seasonal allergic conjunctivitis (group c). Patients were asked to discontinue all topical treatments (anti-histamines, vasoconstrictors or sodium cromoglycate) and systemic medications (antihistamines) 2 weeks before biopsy. Those with other inflammatory eye disease, previous ocular surgery or trauma, were excluded, as were those with perennial allergic symptoms.

Conjunctival biopsy and processing

Following instillation of topical oxybuprocaine (Chauvin Pharmaceuticals, Romford, UK) a snip biopsy was taken

Table 2. The percentage of mast cells which contained each cytokine by co-localization, per patient

		IL-4 (3H4)	IL-4 (4D9)	IL-5	IL-6	IL-8	TNF α
Normals	Median	14	25	0	0	0	0
	Range	0–75	0–54	0–4	0–6	0–0	0–13
In season	Median	38	15	0	0	0	4
	Range	23–86	0–90	0–12	0–25	0–10	0–14
Out of season	Median	16	21	0	0	0	0
	Range	0–77	0–100	0–10	0–13	0–8	0–33

from the superior bulbar conjunctiva, placed immediately in chilled acetone with the protease inhibitors 2 mm phenylmethylsulphonyl fluoride and 20 mm iodoacetamide, and stored overnight at -20°C . The specimens were then processed into the water soluble resin glycolmethacrylate, using the method of Britten *et al.* [34]. Serial sections of 2 μm thickness were cut with an ultramicrotome and stained using monoclonal antibodies 3H4 and 4D9 to IL-4 (Dr C. Heusser, Ciba-Geigy, Basel, Switzerland) [13], MoAb7 to IL-5 and 104B11 to IL-6 (Dr P. Hissey, Glaxo, Greenford UK) [14], 4G9/A5/A7 to IL-8 (Dr I. Lindley, Sandoz Forschungsinstitute, Vienna, Austria) [14], 52B83 to TNF α (Celltech, Slough, UK) [35], AA1 to mast cell tryptase (Dr A. Walls, Clinical Pharmacology, University of Southampton, UK), EG2 to eosinophil cationic protein (Pharmacia Biosystems Ltd, Milton Keynes, UK), and NP57 to neutrophil elastase (Dako Ltd, High Wycombe, UK). Biotinylated rabbit antimouse second stage antibodies (Dako Ltd, High Wycombe, UK) were applied, and immunoreactivity was demonstrated using the streptavidin-biotin peroxidase complex detection method with aminoethylcarbazole as the chromogen, and counterstaining with Mayers haematoxylin. At the same time, two control slides were processed in each case: (a) omitting the primary antibody and (b) using an unrelated monoclonal antibody of the same isotype (M9269, Sigma, Poole UK).

Cell counting and co-localization

Cells were counted using a 1 mm $\times$ 1 mm graticule, at a total magnification of $\times 400$. The total area of each biopsy was calculated using semi-automated image analysis on an Apple Macintosh computer with Colourvision 1.7.4a software (Improvision[®] 1992 and Symantec[®] 1988–91), and

cells counts expressed as numbers/mm². Adjacent sections were examined using the camera lucida system (Leica UK Ltd, Milton Keynes, UK) to localize cytokines to mast cells (i.e. cells staining for tryptase), as well as IL-5 to eosinophils and IL-8 to neutrophils. Only cells with a nucleus were included. Cell counting and co-localization was performed without knowledge of the clinical status of the subject.

Statistical analysis

Cell counts were expressed as medians with ranges, and data from the three groups were compared using the Mann–Whitney *U*-test. A *P*-value of <0.05 was considered significant.

Results

The mean symptom score for in-season patients was 6.75 (range 5–8) which was significantly different from that of the normal and out-of-season groups ($P < 0.0001$): one subject in each of these had a symptom of itch (score = 2) without any other clinical features, while the remainder had a symptom score of zero. All patients with active SAC were biopsied between 1 June and 13 July 1994, during which period the mean local pollen count was 116.7/m³.

Cell counts and co-localization

Mast cell counts are shown in Table 1. Occasional neutrophils were present in the conjunctiva of some subjects from each group. Eosinophils were found in a minority of patients from the normal and in season groups, but not in the out of season group.

To assess the sensitivity of the co-localization technique, serial sections from one biopsy were stained using the

Fig. 1. Three sequential 2 μm sections of conjunctiva stained with: (a) MoAb 4D9 to IL-4; (b) MoAb AA1 to mast cell tryptase; (c) 3H4 also to IL-4, showing the peripheral ring pattern of mast cell staining; and two sequential 2 μm sections of conjunctiva stained with (d) MoAb AA1 to mast cell tryptase and (e) MoAb7 to IL-5. Stained cells are arrowed, magnification is $\times 400$.

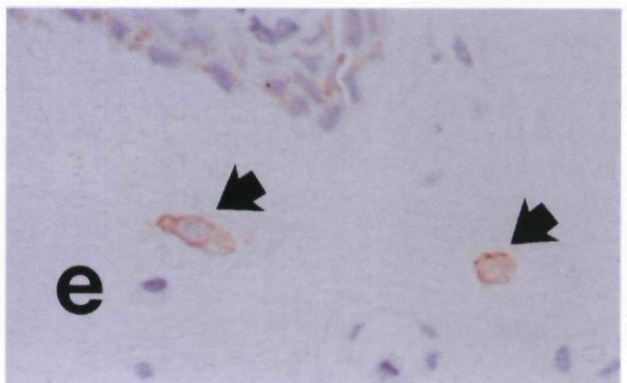
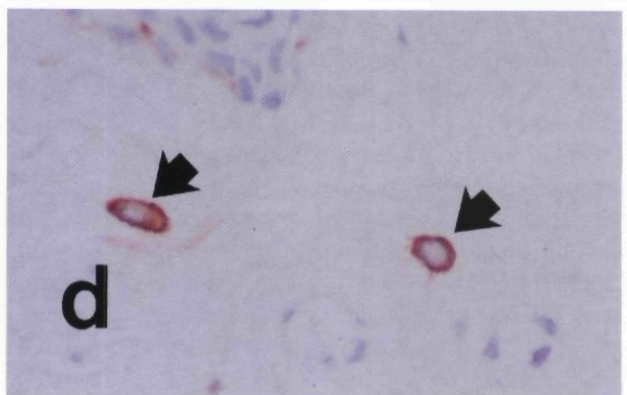
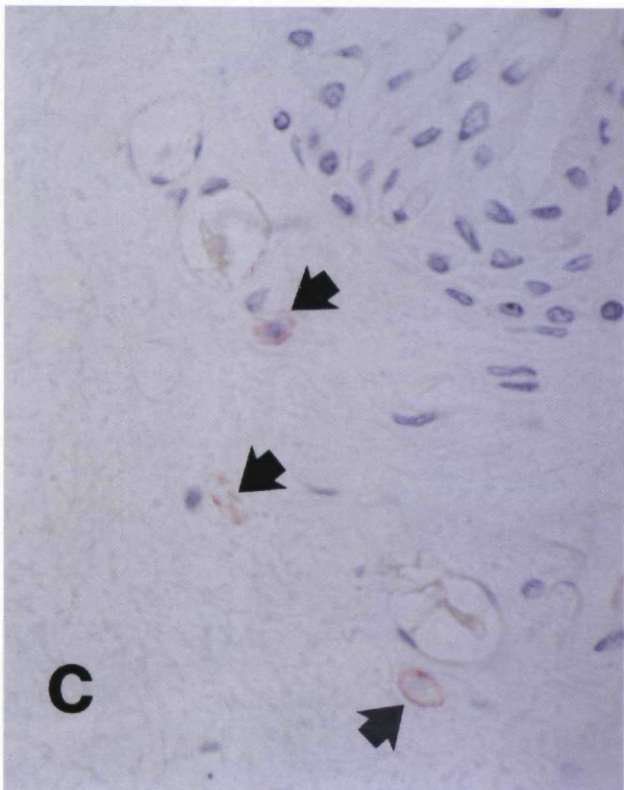
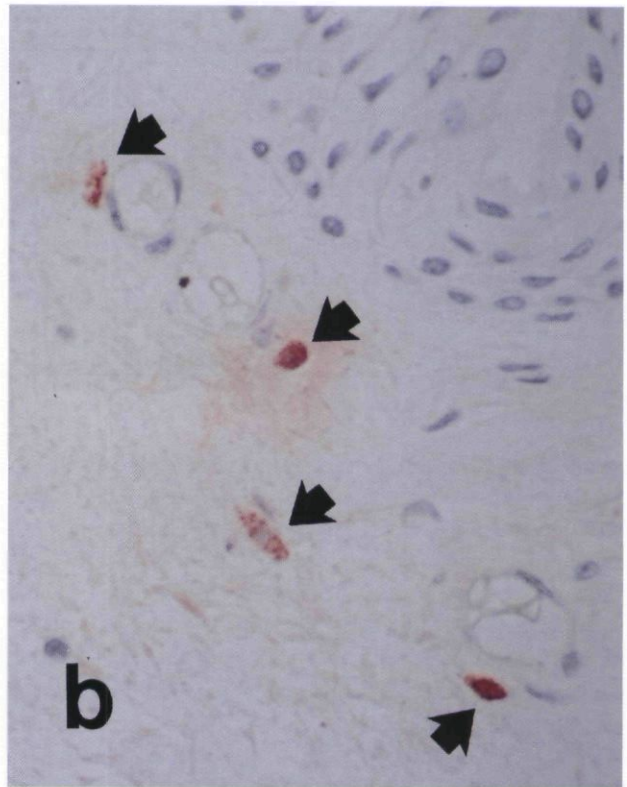
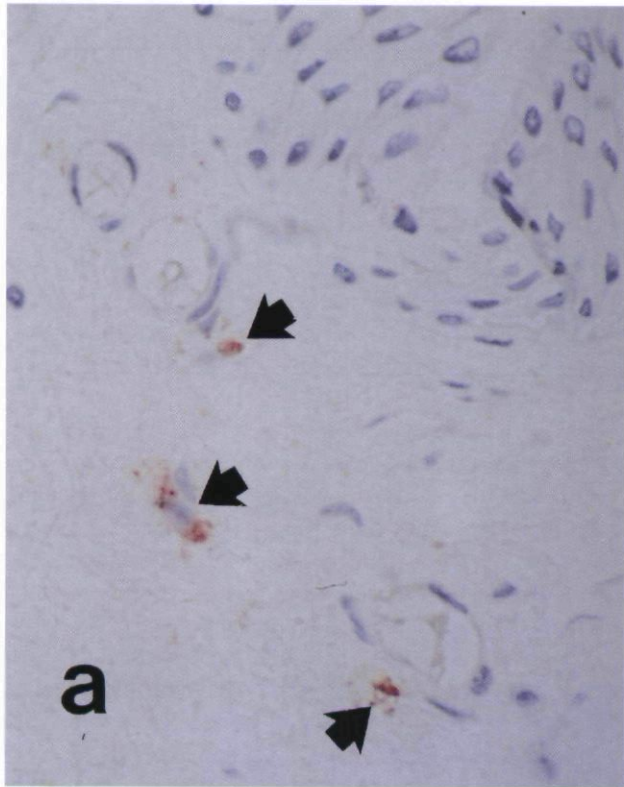


Table 3. The percentage of cytokine-containing cells which were mast cells by co-localization, per patient

		IL-4 (3H4)	IL-4 (4D9)	IL-5	IL-6	IL-8	TNF α
Normal	Median	38	50	0	38	0	42
	Range	0–77	10–75	0–19	9–67	0–0	0–100
In season	Median	60	58	16	23	19	25
	Range	40–82	25–100	0–32	0–100	(n=1)	0–80
Out of season	Median	28	34	22	0	33	17
	Range	0–81	0–71	(n=1)	0–38	(n=1)	0–47

antibody to tryptase. Of the nucleated mast cells identified in one section, 43% were also identified in the next section. No cells were stained in any of the control slides.

The co-localization of cytokines to mast cells is shown in Table 2, and the proportion of cytokine-positive cells which were mast cells is shown in Table 3. The antibody 4D9 localized IL-4 to mast cells in 20 (80%) of the 25 subjects, but there was no significant difference between the groups. By contrast, the antibody 3H4 to IL-4 was localized to mast cells in 19 (76%) subjects, and to a significantly higher proportion of mast cells in SAC than in the out-of-season group ($P < 0.02$). The antibody 3H4 identified mast cells with a characteristic peripheral ring stain, whereas the antibody 4D9 stained the cytoplasm of these cells (Fig. 1a–c). IL-5 was localized to mast cells in three patients (12%) (Fig. 1d–e), one patient coming from each clinical group, but not to eosinophils in any patient. One patient from the in-season group had a markedly raised eosinophil count ($n = 68.6/\text{mm}^2$), and this patient also had the highest mast cell count, the highest neutrophil count, and the highest percentage of interleukin-5 containing mast cells (12% by co-localization). IL-6 was localized to a minority of mast cells in six (24%) patients, distributed over the three groups, while IL-8 was localized to mast cells in two (8%) patients only, one in season and one out of season, and in one of these patients (in season) was localized to neutrophils. There was widespread staining of conjunctival epithelium with this cytokine in all groups. TNF α was localized to mast cells in nine (36%) patients, who came from each of the three groups. When the groups were compared with respect to the proportion of mast cells in each staining for the cytokines IL-5, IL-6, IL-8 and TNF α , no significant differences were found. No staining was seen in any of the negative control slides.

Discussion

In this study we have demonstrated that human conjunctival mast cells store the cytokines IL-4, IL-5, IL-6, IL-8 and

TNF α . In addition, patients with active seasonal allergic conjunctivitis showed a mast cell hyperplasia in which a significantly higher proportion of conjunctival mast cells contained IL-4, as detected with the 3H4 antibody, compared with out-of-season subjects. It is recognized that TH₂ lymphocytes are another potential source of IL-4 and IL-5 in allergic reactions [20], not addressed by this study. However, in allergic rhinitis, immunohistochemistry failed to detect IL-4, IL-5, IL-6 or IL-8 in T cells, possibly due to rapid secretion of cytokines from these cells [14].

SAC is a well defined allergic disease in which direct visualization of the conjunctiva permits an accurate clinical correlate with the cellular processes. While the size and delicacy of this tissue means that only single small biopsies can be taken from individual subjects, the technique of immunohistochemistry on multiple thin sections enables the investigator to examine a range of stored cytokines and cell types in a single specimen. To ensure a high degree of specificity in assigning both cytokines and cell-type to the same individual cells, we included only stained nucleated cells when assessing serial sections. This is likely to underestimate the true proportion of cells stained in the sections, which we attempted to quantify by staining serial sections with the same antibody, AA1 (to tryptase). Our finding that 43% of tryptase + ve cells were identified in an adjacent section indicates the sensitivity of the technique, and implies that in SAC the great majority of mast cells contain IL-4, and that nearly all IL-4-containing cells are mast cells.

As in our previous study [1] we have demonstrated a mast cell hyperplasia in SAC compared with the non-allergic state. Mast cell numbers in out-of-season subjects were also elevated compared with normals, although this did not reach statistical significance (Table 1), which suggests that in SAC mast cell numbers increase and do not return to normal even out of season. An alternative and less likely possibility is that atopy is associated with an increase in conjunctival mast cell numbers which is independent of ocular allergy. The control of mast cell numbers, as well as of mast cell migration into the epithelium, is clearly of great importance

in the pathogenesis of allergic conjunctivitis. Recent work has suggested a major role for stem cell factor (*c-kit* ligand), which promotes mast cell proliferation, maturation, migration and mediator release [36], although the source of stem cell factor in the conjunctiva remains to be established.

The contribution of neutrophils and eosinophils to the symptomatology of seasonal allergic conjunctivitis has not been determined. Our results suggest that significant symptoms can occur without the participation of these cells, which were infrequent even in the presence of increased expression of IL-4 in mast cells. A likely explanation is that our patients had relatively mild SAC (with a mean symptom score of 6.75 out of a possible maximum of 11), in which allergic inflammation can be attributed to mast cell degranulation alone [37], whereas at higher pollen levels than those experienced by our patients, enhanced cytokine release leads to the influx of neutrophils and eosinophils [8] and further exacerbation of the clinical features. Eosinophils are of major importance in vernal keratoconjunctivitis [38], a more serious form of allergic eye disease, and the present study indicates that conjunctival mast cells may be a source of cytokines (IL-4, IL-5, IL-8, TNF α) involved in eosinophil recruitment.

Our finding that in SAC, compared with the out-of-season state, a significantly greater proportion of mast cells contained IL-4 as detected with the 3H4 antibody (whereas the proportion detected with 4D9 antibody was unchanged) is paralleled in allergic rhinitis and may reflect a conformational change in IL-4 occurring during the switch from cytoplasmic storage (4D9-staining) to active secretion, revealed by the characteristic peripheral ring stain (3H4-staining – Fig. 1) [14]. Recently, Fujishima *et al.* have described elevated IL-4 levels in tears from patients with SAC and vernal keratoconjunctivitis [39]. Since mast cells appear to be an important source of IL-4 in SAC, this gives them a much greater role in this disease than previously considered, in which IgE-dependent activation causes further mast cell-driven upregulation of the allergic response, and may include the induction of local IgE synthesis by B lymphocytes without the participation of T cells [40].

IL-5 and IL-6 were localized to a small minority of mast cells in a few patients, in contrast to the much higher values found in normal and allergic nasal mucosa [14], which may reflect functional differences between these mucosal sites. In allergic rhinitis, the much higher proportion of IL-5-containing mast cells was associated with a marked eosinophil infiltration.

This study found that conjunctival mast cells can store immunoreactive IL-8, which was detectable in a small minority of mast cells in two subjects. By contrast, there was widespread staining of conjunctival epithelium in all groups for this cytokine, as previously described in both nasal and bronchial mucosa [14,28]. It has been suggested

that the biological activity of IL-8 and other pro-inflammatory cytokines depends not only on their concentration in tissues, but also their binding to matrix molecules such as glycosaminoglycans [28].

In our study TNF α was localized to conjunctival mast cells in normal and allergic subjects, with no significant difference between the groups with respect to the proportions of mast cells stained. A major function of this cytokine is the recruitment of neutrophils and eosinophils, but these cells were present in low numbers in our biopsies and not increased in SAC.

We have shown that conjunctival mast cells, in common with those at other sites involved in allergic reactions, store several multifunctional cytokines. This suggests a wide-ranging role for mast cells in regulating the normal conjunctival microenvironment. In allergic subjects it provides a mechanism by which activation of these cells may lead not only to the immediate symptoms of SAC but also, through the IgE-dependent release of stored cytokines, to a rapid upregulation of conjunctival inflammation in all forms of ocular allergy.

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