

Seasonal allergic conjunctivitis is accompanied by increased mast cell numbers in the absence of leucocyte infiltration

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Summary

Background Seasonal allergic conjunctivitis (SAC) is the most common allergic disease to affect the eye, occurring alone or in association with allergic rhinitis. Infiltration with mast cells and eosinophils is characteristic of the chronic forms of allergic conjunctivitis such as vernal and atopic keratoconjunctivitis, and these cell types also contribute significantly to allergic inflammation in the skin. Indirect evidence for a similar pattern of cellular events in SAC comes from studies which demonstrate raised eosinophil and neutrophil numbers in conjunctival scrapings and elevated levels of mast cell tryptase in tears following allergen challenge.

Objective To directly characterize the inflammatory cell infiltrate in SAC and to determine its clinical relevance.

Methods We employed specific immunohistochemical staining to count mast cells, eosinophils and neutrophils in the conjunctival epithelium and lamina propria of eight atopic patients with SAC in, and 12 SAC patients out of the hay fever season. Sixteen patients with no history of ocular allergy were used as control subjects.

Results Mast cells were absent from normal epithelium. During the pollen season median mast cell numbers in the lamina propria were found to be increased by 61% in patients with SAC compared with normals ($P = 0.012$). Eosinophils were found in the lamina propria in less than half of the symptomatic patients with SAC and in only three patients were eosinophils present in the epithelium. The neutrophil numbers in the lamina propria of patients with SAC tended to be higher than normals but these changes did not reach statistical significance.

Conclusion These data based on the direct assessment of conjunctival tissue provide evidence that symptoms occur in SAC in the absence of detectable recruitment of eosinophils or neutrophils. This suggests that this disorder is related to mast cell-mediated changes.

Keywords: mast cell, eosinophil, neutrophil, allergy, conjunctivitis, granulocyte, eye

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Introduction

Seasonal allergic conjunctivitis is the most common ocular allergic disease. Although not sight threatening, symptoms of itch, tearing and mucus discharge may be extremely

irritating and are a frequent source of patient self-referral to family practitioners. Exacerbations of symptoms occur with peaks of pollen release and in areas of high atmospheric pollution [1], and symptoms remit when the pollen count falls.

Mast cell activation is thought to be central to the genesis of this acute allergic response. High affinity FcεR1 receptors are aggregated by multivalent allergen cross-linking cell

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surface IgE. This leads to cell activation and the release of histamine and newly synthesized lipid mediators including prostaglandin D₂ and the sulphidopeptide leukotriene (LT), LTC₄. Topical application of several of these agents reproduces the signs of allergic conjunctivitis [2,3], whilst histamine, leukotrienes, and the unique mast cell neutral protease, tryptase have been demonstrated in the tears of atopic individuals following allergen challenge [4–6]. Mast cell release of histamine and leukotrienes may also contribute to these inflammatory events by recruitment of eosinophils and neutrophils [7] and more recently, mast cells have also been shown to influence the allergic inflammatory response by the secretion of a range of cytokines [8] including interleukin-4 (IL-4) [9] and TNF α [10]. We have recently immunolocalized IL-4, IL-5, IL-6, IL-8 and TNF α to human conjunctival mast cells [11]. This capacity to rapidly release cytokines, which can occur prior to T cell ingress, confers upon the mast cell the ability to orchestrate the immune response as well as participation in mediating the acute allergic response.

Evidence provided by tear assays suggests that there is local IgE production in the conjunctiva. Pollen specific IgE has been demonstrated in the tears and sera of affected individuals with a highly significant correlation between the former with symptoms of SAC [12]. The observation that IgE production by B cells may be induced by mast cells independently of T cell help [13] suggests that mast cells may be involved in the autoregulation of IgE production through CD40/CD40 ligand dependent mechanisms in the presence of IL-4, independent of T-cell activation.

Allergic diseases at other mucosal sites are now recognised to occur in association with a characteristic pattern of granulocyte-rich inflammation, involving neutrophils in acute responses and a striking predominance of eosinophils in the chronic disease state [14]. The role of the neutrophil in allergic conjunctivitis is however, unclear. Neutrophils but not eosinophils have been demonstrated in the normal human conjunctiva by Giemsa staining [15], and neutrophil numbers have been found to be elevated in conjunctival scrapings taken from atopic subjects after high dose topical allergen challenge [16]. Histological studies performed on conjunctiva obtained from patients with the chronic allergic conjunctivides, atopic (AKC) and vernal (VKC) keratoconjunctivitis however, fail to demonstrate significant increases in neutrophil numbers [17,18].

The presence of large numbers of eosinophils in the lamina propria is characteristic of AKC and VKC [17,18] as is the migration of these cells into the epithelium. The finding of the cytotoxic cationic proteins, eosinophil granule major basic protein (MBP), Charcot-Leydon crystal protein [19] and eosinophil cationic protein (ECP) in the tears of patients with VKC [20] also strongly suggests eosinophil recruitment and subsequent activation in these conditions.

Eosinophils have been demonstrated in conjunctival scrapings taken from patients with symptomatic SAC but may be absent [21]. The failure to recover eosinophils from conjunctival scrapings should not preclude the diagnosis of SAC.

These observations imply a role for mast cells, eosinophils and neutrophils in SAC. The relationship however, between the cellular constituents of conjunctival scrapings, cell and mediator findings in the tears, and the actual cellular events taking place in the conjunctiva remain to be determined. We have investigated the cell populations in the conjunctival lamina propria and epithelium to determine the cellular basis of the symptomatology in this common ocular disorder.

Materials and methods

Subjects

Ethical approval for this study was granted by the Southampton Joint Ethics Committee. All patients gave written informed consent. Patients were interviewed and examined with a slit lamp biomicroscope. The scoring system devised by Abelson *et al.* [22] was used to evaluate all the subjects recruited. Patients were diagnosed as suffering from SAC on the basis of their history, ocular examination and positive skin-prick test to mixed grass pollen. All patients refrained from the use of systemic or topical medication for at least 2 weeks prior to their entry into the study. All patients were free from other ocular disease and no patient wore contact lenses. Conjunctival biopsies were obtained from eight atopic patients with SAC in season (five male, three female, mean age 50.1 years, range 23–76) 12 atopic patients with SAC out of season (six male, six female, mean age 42 years, range 19–74) and 16 non-atopic controls (eight male, eight female, mean age 57 years, range 21–89). The patients studied within the pollen season were biopsied between 1st June and 15th July 1994 when the mean pollen count was 116.7 grains m⁻³. The patients studied outside of the pollen season were biopsied between November and February of 1992 and 1994. There was no statistical difference between the groups for age or sex.

Skin-prick tests

The following panel of allergens was used: six grasses, *A. elatior*, *D. glomerata*, *F. pratensis*, *L. perenne*, *P. pratense* and *P. pratensis*, house dust mite (*D. pteronyssinus*) and cat dander (Soluprick ALK Laboratorium, Denmark). Each drop was applied to the forearm and the skin punctured with a 23G sterile needle. The site of each allergen was marked with a ball point pen. Histamine dihydrochloride (Soluprick) was used as a positive control and diluent

without allergen as a negative control. After 15 min the diameter of the weal was measured in millimeters. A weal diameter of 3 mm greater than the negative control was regarded as positive.

Conjunctival biopsy, tissue fixation and processing

Snip biopsies from the upper bulbar conjunctiva were obtained after topical application of the local anaesthetic, oxybuprocaine hydrochloride (Chauvin Pharmaceuticals Ltd, UK). Topical broad spectrum antibiotic drops were applied immediately post-operatively and for the next 48 h.

Biopsies were immersed in chilled acetone containing protease inhibitors (2 mM phenylmethylsulphonyl fluoride and 20 mM iodoacetamide) and stored overnight at -20°C . The biopsies were then processed into a water soluble resin, glycol methacrylate (GMA) as described previously [23]. The blocks were polymerized overnight at 4°C and stored at -20°C in airtight containers containing silica resin.

Immunohistochemistry

Two micrometre sections were cut by ultramicrotomy and floated on to 0.2% ammonia solution in water for $1\frac{1}{2}$ min, picked up on poly L-lysine coated slides and air-dried for between 1 and 3 h. Endogenous peroxidase activity was blocked by treatment with sodium azide 0.1% and hydrogen peroxide 0.003% for 30 min. Non-specific binding sites were blocked by incubation in tissue culture medium (Dulbecco's and 20% fetal calf serum) for 30 min. Primary antibodies were applied overnight at room temperature. The following mouse IgG1 monoclonal antibodies were used at previously titrated optimal dilutions: AA1 to mast cell tryptase (Dr A. F. Walls, Clinical Pharmacology, Southampton University, UK), EG2 to ECP (Pharmacia Biosystems Ltd, Milton Keynes, UK) and NP57 to neutrophil elastase (Dako Ltd, High Wycombe, UK). Control slides were treated identically, one with omission of the primary antibody, the other with a non-specific IgG1 monoclonal antibody at the highest concentration of primary antibody used (M9269 Sigma, Poole, UK). Biotinylated second stage antibodies (Dako Ltd, High Wycombe, UK) were applied for 2 h followed by detection with streptavidin-biotin-peroxidase complexes (Dako Ltd, UK) using aminoethylcarbazole to give a fine red reaction product for light microscopy. Slides were counterstained with Mayers haematoxylin, mounted in Crystal Mount (Biomedica, Bournemouth, UK) baked at 80°C for 10 min and coverslipped with DPX mounting medium.

Quantification and statistical analysis

Cells were counted without knowledge of their original

source. Only nucleated cells were counted, and all cells within each biopsy were counted at a magnification of $\times 400$. Twenty per cent of the slides were re-labelled and re-counted, again without knowledge of their source. The area of lamina propria (mm^{-2}) and length of the epithelium (mm) was measured using semiautomated image analysis with Colourvision 1.7.4a software (ImproVision, Warwick, UK and Symantec, Cupertino, CA, USA) on an Apple Macintosh computer. Data were analysed using the Mann-Whitney *U*-test to explore the relationship between data sets. A *P*-value of $P = 0.05$ was taken to be significant. Using the method of Bland [24], a coefficient of repeatability (twice the standard deviation of the log transformed differences in counts) was calculated for the recounted slides.

Results

All patients with SAC in season were symptomatic with itch, redness and tearing. Patients with SAC in season had a mean symptom score of 6.75 (range 5–8), all patients with SAC out of season and the normal controls had symptom scores of zero except one subject in each group whose score was two due to itch. Analysis of symptom scores showed significant differences between SAC in season and normals ($P = 0.0001$) and SAC patients in and out of season ($P = 0.0002$). No difference in scores existed between normals and SAC patients out of season ($P = 0.94$). The greatest mast cell counts were observed in the patients with maximal symptom scores.

Clear immunostaining was present in mast cell, eosinophil and neutrophil cytoplasm with no positive staining obtained in any of the negative control slides (Fig. 1). Two mast cells were observed in the epithelium of one patient with SAC in season (Fig. 1A). No mast cells or eosinophils were detected in the epithelium of the normal group and in each of two subjects one neutrophil was observed. No neutrophils were detected in the epithelium of symptomatic patients in season.

In only three of these patients were eosinophils observed in the epithelium, a finding which did not reach statistical significance.

The median mast cell density in normal lamina propria was 14.9 (range 3.4–28.7) cells mm^{-2} . Compared with normals mast cell numbers were elevated significantly in SAC in season (median 23.9, range 16.5–52.5 cells mm^{-2}) ($P = 0.012$). Although no statistical difference existed between the mast cell count in and out of season in SAC, the mast cell count in SAC patients out of season (median 20.2, range 10.3–46.4 cells mm^{-2}) ($P = 0.071$) was not significantly greater than for normals.

Low numbers of neutrophils and eosinophils with wide inter-subject variation were found in the conjunctival

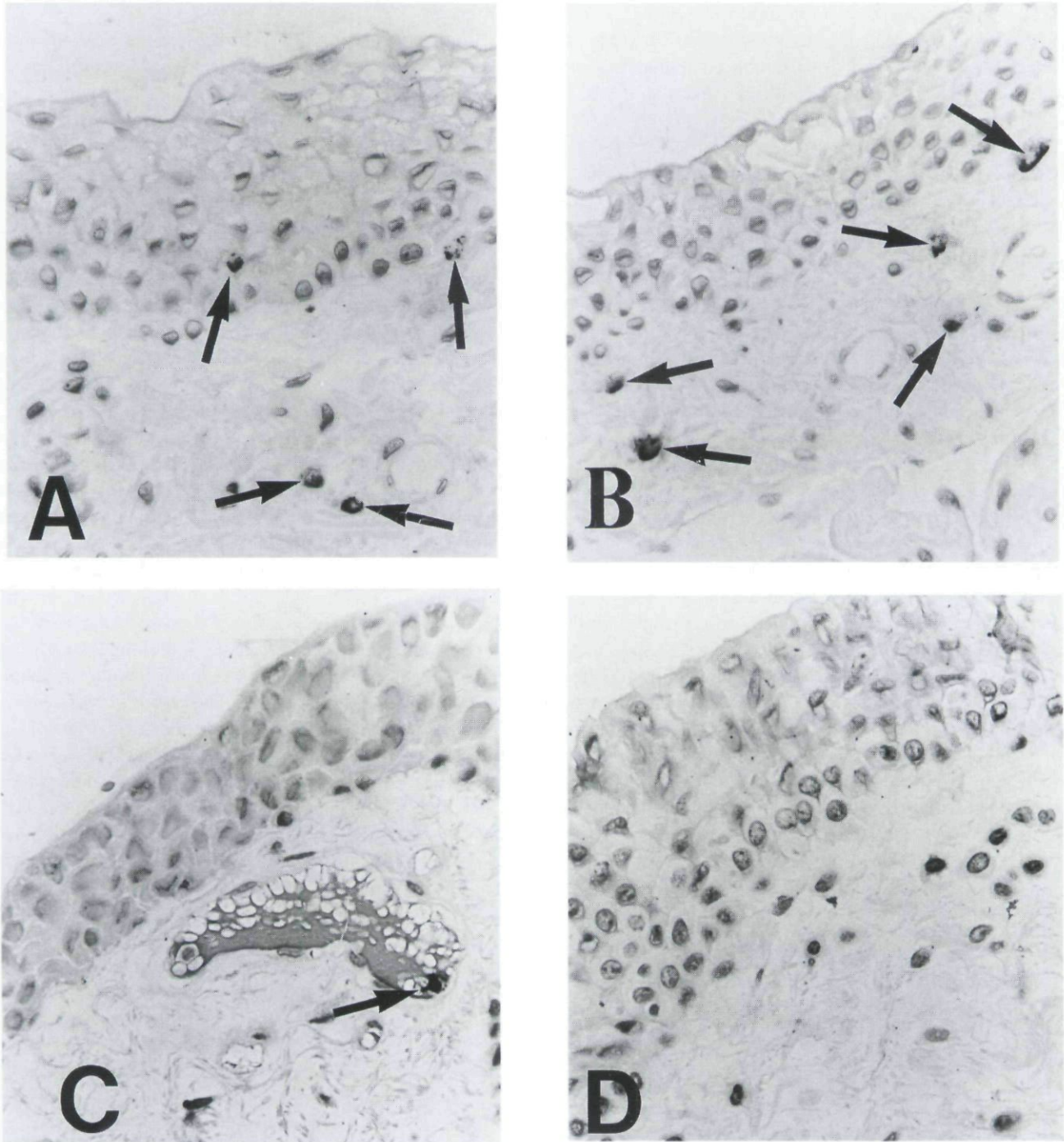


Fig. 1 Series of 2 µm sections of bulbar conjunctiva. Arrows show immunohistochemical staining of granulocytes within each section. Magnification X340. (A) Mast cells labelled with MoAb AA1 to mast cell tryptase. (B) Eosinophils labelled with MoAb EG2 to eosinophil cationic protein. (C) Intravascular neutrophil labelled with MoAb NP57 to neutrophil elastase. (D) Negative control slide using IgG1 isotype monoclonal antibody, no cellular staining is evident.

lamina propria particularly in the SAC subjects. The median eosinophil count was 0 (range 0–2.7) cells mm^{-2} in normals and 0 (range 0–68.6) cells mm^{-2} in subjects with SAC in season. Similarly, there was no difference in eosinophil numbers between normal and SAC subjects out of season.

There was no significant difference in neutrophil counts in the lamina propria of patients with SAC (median 1.8, range 0–13.3 cells mm^{-2}) in season compared with both normal (median 0.4, range 0–6.8 cells mm^{-2}) and SAC out of season (median 0.5, range 0–4.2 cells mm^{-2}) (Fig. 2).

By recounting the same slides a coefficient of repeatability of 3.1 cells mm^{-2} was calculated indicating that the counts were reproducible and reliable.

Discussion

This study demonstrated a significant increase in mast cell numbers in the lamina propria of subjects with SAC during the hay fever season. Although mast cell numbers were higher than normals in subjects with SAC out of season this change did not achieve statistical significance. The use of GMA resin allows thin sections of tissue to be cut with excellent preservation of morphology, maintenance of tissue antigens and accurate localisation of cell markers [23]. These features increase both the sensitivity and specificity of cell counts compared with immunohistochemistry performed on frozen sections which are typically cut at 6–8 μm . The use of the monoclonal antibodies AA1 to mast cell tryptase, EG2 to ECP and NP57 to neutrophil elastase allowed specific labelling of these cell types. Despite the use of highly sensitive and selective enzyme-immunohistochemistry, we could not detect any consistent change in granulocyte populations. The technique of counting only nucleated cells led to counts lower than those reported elsewhere [15], even allowing for correction for area rather than volumetric counts. We felt that this method

avoided the possibility of counting the same cell twice particularly when cells were partially degranulated, and hence led to greater reproducibility. To avoid sampling errors by counting selective areas within a section of tissue, the whole section was examined and all cells counted.

Both mast cells and T cells are present in conjunctival tissue but unlike T cells, mast cells are able to store as well as release a TH_2 -like panel of cytokines [25] without prior exposure to IL-4. Mast cells have been noted to shift phenotype from cells containing the proteases tryptase and chymase (MC_{TC}) to predominantly those containing tryptase only (MC_{T}) in symptomatic patients with SAC [26]. This may reflect the selective recruitment of MC_{T} or the preferential maturation of these cells in a microenvironment where cells are exposed to repeated allergen exposure, degranulation and subsequent regranulation. Some evidence in rhinitic subjects suggests that mast cell phenotype may regulate the spectrum of cytokines secreted [27] although the concept of discrete mast cell subsets with particular cytokine secretory patterns has yet to be explored.

We found no mast cells or eosinophils in the conjunctival epithelium of our normal subjects. This is consistent with previous morphological reports [15] although evidence from conjunctival cytology is conflicting. Some workers have reported low numbers of eosinophils present in scrapings obtained from normal subjects [28], but others have failed to demonstrate eosinophils in conjunctival scrapings obtained from patients with VKC even when they were demonstrated histologically in the epithelium [21]. The lack of increase in eosinophil numbers in the conjunctival lamina propria from subjects with SAC in season is consistent with the mild nature of the inflammation and the absence of sight threatening complications. Eosinophil activation leads to the release of the arginine rich secondary granule proteins MBP, ECP and eosinophil peroxidase (EPO). MBP and ECP display cytotoxicity for human respiratory epithelial cells [29] and MBP has been shown to delay corneal wound healing in vitro [30], and to be deposited in the corneal ulcers associated with VKC [31].

Low numbers of neutrophils have been described previously in the epithelium of normal conjunctival biopsies [15] although we found only solitary neutrophils in the epithelium of two subjects. Continued exposure of the conjunctiva to the atmosphere and commensal bacteria whose products are potent chemoattractants for neutrophils [32], may explain the presence of neutrophils in an otherwise healthy tissue. In patients with SAC studied in season, we found no significant increase in neutrophil numbers in the lamina propria when compared to SAC out of season or normal subjects. Overall our evidence suggests that neutrophil infiltration does not play a significant role in SAC during the seasonal period of allergen exposure.

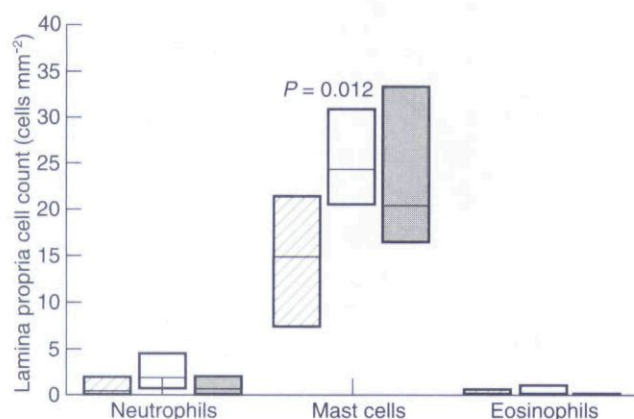


Fig. 2 Medians and interquartile ranges for cell counts in the conjunctival lamina propria from normal controls, symptomatic patients with SAC and SAC patients out of season.

In summary, this study provides the first account of the tissue changes in conjunctival mast cell, eosinophil and neutrophil numbers in SAC. We have shown a significant rise in mast cell numbers in the lamina propria but not epithelium of symptomatic subjects with SAC, but no significant increase in either neutrophil or eosinophil numbers either in the lamina propria or epithelium. Our observations suggest that there is a fundamental pathological difference between SAC, in which sight threatening complications do not occur, and the chronic forms of allergic conjunctivitis in which a principally eosinophilic inflammation is associated with the potential for corneal blindness.

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References

- 1 Varney V. Hayfever in the United Kingdom. *Clin Exp Allergy* 1991; 21:757–62.
- 2 Ciprandi G, Buscaglia S, Pesce GP, Bagnasco M, Canonica GW. Ocular challenge and hyperresponsiveness to histamine in patients with allergic conjunctivitis. *J Allergy Clin Immunol* 1993; 91:1227–30.
- 3 Woodward DF, Ledgard SE. Comparison of leukotrienes as conjunctival microvascular permeability factors. *Ophthalmic Res* 1985; 17:318–20.
- 4 Proud D, Sweet J, Stein P et al. Inflammatory mediator release on conjunctival provocation of allergic subjects with allergen. *J Allergy Clin Immunol* 1990; 85:896–905.
- 5 Bisgaard H, Ford-Hutchinson AW, Charleson S, Taudorf E. Production of leukotrienes in human skin and conjunctival mucosa after specific allergen challenge. *Allergy* 1985; 40:417–23.
- 6 Butrus SI, Ochsner KI, Abelson MB, Schwartz LB. The level of tryptase in human tears. *Ophthalmology* 1990; 97:1678–83.
- 7 Woodward DF, Spada CS, Hawley SB, Nieves AL. Conjunctival eosinophil infiltration evoked by histamine and immediate hypersensitivity: Modification by H1- and H2-receptor blockade. *Invest Ophthalmol Vis Sci* 1986; 27:1495–503.
- 8 Gordon JR, Burd PR, Galli SJ. Mast cells as a source of multifunctional cytokines. *Immunol Today* 1990; 11:458–64.
- 9 Bradding P, Feather IH, Howarth P et al. Interleukin 4 is localised to and released by human mast cells. *J Exp Med* 1992; 176:1381–6.
- 10 Gordon JR, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF- α /cachectin. *Nature* 1990; 346:274–6.
- 11 MacLeod JDA, Anderson DF, Baddeley SM, McGill JI, Holgate ST, Roche WR. Immunolocalization of cytokines to mast cells in normal and allergic conjunctiva. *Clin Exp Allergy*, in press.
- 12 Hoffmann-Sommergruber K, Ferreira FD, Ebner C et al. Detection of allergen-specific IgE in tears of grass pollen-allergic patients with allergic rhinoconjunctivitis. *Clin Exp Allergy* 1996; 26:79–87.
- 13 Gauchat JF, Henchoz S, Mazzei G et al. Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature* 1993; 365:340–3.
- 14 Djukanovic R, Wilson JW, Britten KM et al. Quantification of mast cells and eosinophils in the bronchiol mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Res Dis* 1990; 142:863–71.
- 15 Allansmith MR, Greiner JV, Baird RS. Number of inflammatory cells in the normal conjunctiva. *Am J Ophthalmol* 1978; 86:250–9.
- 16 Bonini S, Bonini S, Vecchione A, Naim DM, Allansmith MR, Balsano F. Inflammatory changes in conjunctival scrapings after allergen provocation in humans. *J Allergy Clin Immunol* 1988; 82:462–9.
- 17 Allansmith MA, Baird RS, Greiner JV. Vernal conjunctivitis and contact lens-associated giant papillary conjunctivitis compared and contrasted. *Am J Ophthalmol* 1979; 87:544–55.
- 18 Foster CS, Rice BA, Dutt JE. Immunopathology of atopic keratoconjunctivitis. *Ophthalmology* 1991; 98:1190–6.
- 19 Udell IJ, Gleich GJ, Allansmith MR, Ackerman SJ, Abelson MB. Eosinophil granule major basic protein and Charcot-Leyden crystal protein in human tears. *Am J Ophthalmol* 1981; 92:824–8.
- 20 Leonardi A, Borghesan F, Faggian D, Secchi A, Plebani M. Eosinophil cationic protein in tears of normal subjects and patients affected by vernal keratoconjunctivitis. *Eur J Allergy Clin Immunol* 1995; 50:610–3.
- 21 Abelson MB, Udell IJ, Weston JH. Conjunctival eosinophils in allergic ocular disease. *Arch Ophthalmol* 1983; 101:631.
- 22 Abelson MB, Chambers WA, Smith LM. Conjunctival allergen challenge. A clinical approach to studying allergic conjunctivitis. *Arch Ophthalmol* 1990; 108:84–8.
- 23 Britten KM, Howarth PH, Roche WR. Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies. *BiotechHistochem* 1993; 68:271–80.
- 24 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; 86:307–10.
- 25 Plaut M. Antigen-specific lymphokine secretory patterns in atopic disease. *J Immunol* 1990; 144:4497–500.
- 26 Baddeley SM, Bacon AS, McGill JI, Lightman SL, Holgate ST, Roche WR. Mast cell distribution and neutral protease expression in acute and chronic allergic conjunctivitis. *Clin Exp Allergy* 1995; 25:41–50.
- 27 Bradding P, Okayama Y, Howarth PH, Church MK, Holgate ST. Heterogeneity of human mast cells based on cytokine content. *J Immunol* 1995; 155:297–307.
- 28 Kari O, Haahtela T. Conjunctival eosinophilia in atopic and non-atopic external eye symptoms. *Acta Ophthalmol* 1992; 70:335–40.
- 29 Venge P, Dahl R, Fredens K, Peterson CGAD. Epithelial injury by human eosinophils. *Am Rev Res Dis* 1988; 138:S54–7.

- 30 Trocme SD, Gleich GJ, Kephart GM, Zieske JD. Eosinophil granule major basic protein inhibition of corneal epithelial wound healing. *Invest Ophthalmol Vis Sci* 1994; 35:3051-6.
- 31 Trocme SD, Kephart GM, Bourne WM, Buckley RJ, Gleich GJ. Eosinophil granule major basic protein deposition in corneal ulcers associated with vernal keratoconjunctivitis. *Am J Ophthalmol* 1993; 115:640-3.
- 32 Schiffmann E, Corcoran BA, Wahl SM. N-formylmethionyl peptides as chemoattractants for leucocytes. *Proc Natl Acad Sci USA* 1975; 72:1059-62.

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