

Eosinophil surface antigen expression and cytokine production vary in different ocular allergic diseases

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Background: The pathophysiology of chronic ocular allergic disease is not well understood. An eosinophil infiltrate is characteristic of such disease and eosinophil activity can be related to disease severity and to keratopathy, the most serious complication. Recently, eosinophils have been shown capable of cytokine production, particularly in allergic disease, although the disease-specific cytokine spectrum of tissue eosinophils is unknown.

Objectives: We sought to determine eosinophil numbers (absolute numbers and percentage of total leukocytes), cell surface antigen expression, and cytokine production in conjunctiva in chronic allergic eye disease and their relationship to corneal involvement.

Methods: Ultrathin sections of conjunctiva were examined by tissue staining and by 1- and 2-color immunohistochemistry.

Results: Eosinophil numbers were greater in giant papillary conjunctivitis (GPC) and vernal keratoconjunctivitis (VKC) and not related to corneal involvement. The eosinophil expression of the cell surface antigens intercellular adhesion molecule-1, CD4, IL-2R, and HLA-DR was greater in atopic keratoconjunctivitis (AKC) and VKC, the disorders with corneal disease, than in GPC, in which the cornea is not involved. For most cytokines, localization to eosinophils was greater for VKC and AKC than for GPC. RANTES, TGF- β , and TNF- α localized to eosinophils in all disorders. Variations in the pattern of eosinophil-cytokine localization were found. In VKC IL-3, IL-5, IL-6, and GM-CSF were prominent; in GPC IL-5 was prominent; and in AKC IL-4, IL-8, and GM-CSF were prominent.

Conclusions: Chronic ocular allergic disorders affecting the cornea are distinguished from disorders that do not do so by greater expression of eosinophil surface antigens (which may imply greater cell activation) and differences in cytokine localization to eosinophils. These differences may be secondary to the variations in T-cell subsets or a primary phenomenon. Changes in eosinophil function, rather than cell numbers, may be important in clinical variations, such as keratopathy, and may allow future therapeutic exploitation. (*J Allergy Clin Immunol* 1998;102:821-30.)

Key words: Allergy, adhesion molecules, cytokines, MHC, immunochemistry

The term *ocular allergic disease* covers a group of ocular surface disorders associated with atopy and distinguished by ocular itching, discharge, and conjunctival papillae. Seasonal allergic (hay fever) conjunctivitis and perennial allergic conjunctivitis have mild ocular signs and do not affect the cornea.¹ Vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) cause giant papillae and scarring of the conjunctiva and, by means of corneal involvement (keratopathy), can be sight-threatening.^{2,3} Contact lens-related giant papillary conjunctivitis (GPC) is clinically and histologically similar to AKC and VKC⁴ except that, for unknown reasons, serious keratopathy does not occur.⁵

A type-I hypersensitivity reaction is believed to underlie seasonal allergic conjunctivitis and perennial allergic conjunctivitis.⁴ The situation in AKC, VKC, and GPC is more complex, although a number of lines of evidence support a role for type-I hypersensitivity in these disorders.^{6,7} T cell-mediated inflammation, with a T_{H2}-like pattern of cytokine production in VKC and GPC and a T_{H1}-like pattern in AKC, appears to be important.⁸⁻¹⁰ Cytokine production by non-T cells (eg, mast cells or epithelial cells) has been implicated in nonocular allergic disease,^{11,12} and recent work shows that such cytokine production is possible in the conjunctiva.¹³⁻¹⁶

An eosinophil infiltrate is characteristic of ocular allergic disease and is virtually pathognomonic.¹⁷ Eosinophils are able to produce a wide range of cytokines, which may influence their own activity and that of other leukocytes.^{18,19} Eosinophils are also able to produce multiple inflammatory mediators, including phospholipase A₂ products, toxic oxygen metabolites, and granule proteins.²⁰ There is some evidence supporting a link between eosinophils and the development of allergic corneal disease. The levels of granule proteins in tears and serum correlate with disease severity,^{21,22} and tissue deposition occurs in areas of keratopathy.²³ These proteins do have some toxic effect on the corneal epithelium,²⁴ but their presence may simply be a reflection of intense eosinophil activity and not the primary cause of the associated corneal disease.

The aims of this study were 2-fold. First, we aimed to perform a descriptive study to identify which cytokines are produced by eosinophils in serious ocular allergy,

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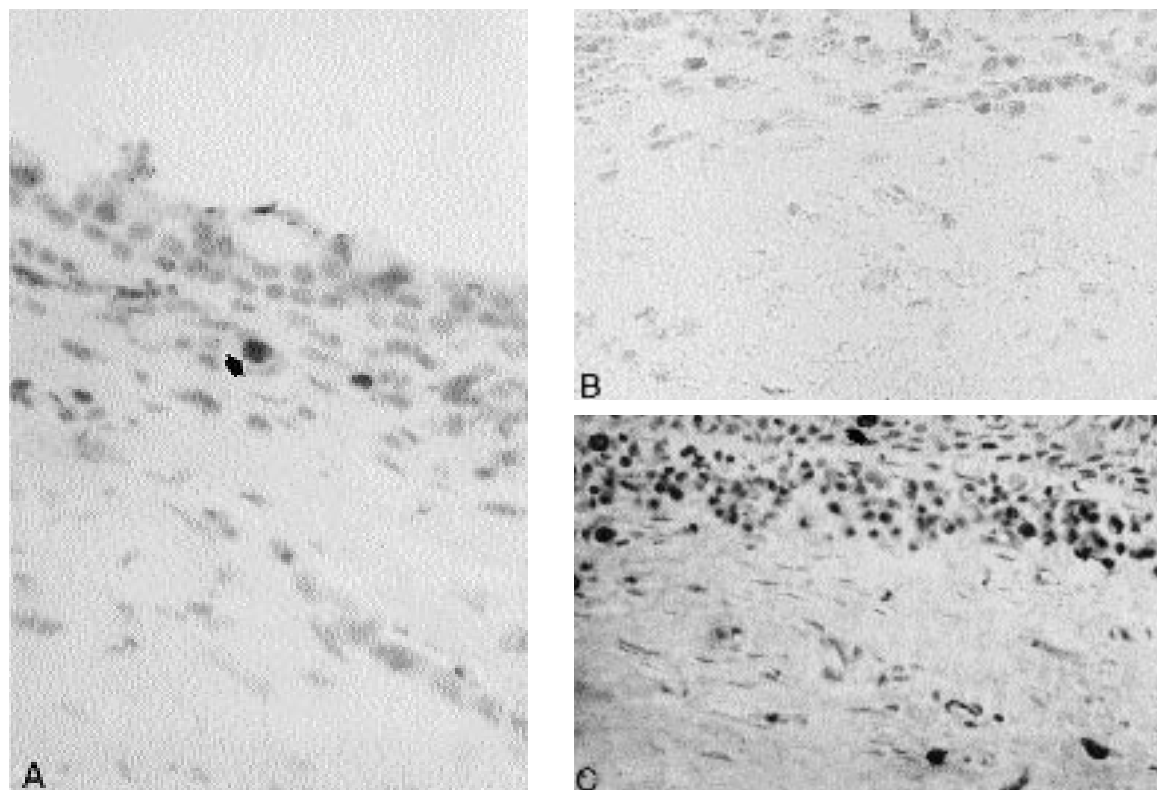


FIG 1. **A**, Example of AEC single-positive cell; red AEC deposition demonstrating IL-4 immunoreactivity (*black arrow*). **B**, No immunoreactivity in control with unrelated antibody. **C**, DAB single positive cells; eosinophil cationic protein positivity in eosinophils (2- μ m sections, $\times 100$ to $\times 200$ magnification, hematoxylin counterstain).

Abbreviations used

AEC:	Amino-ethyl carbazole
AKC:	Atopic keratoconjunctivitis
DAB:	Diaminobenzamine
GPC:	Giant papillary conjunctivitis
ICAM-1:	Intercellular adhesion molecule-1
RANTES:	Regulated upon activation, normal T cells expressed and secreted
TGF:	Transforming growth factor
VKC:	Vernal keratoconjunctivitis

about which almost nothing is known, and to determine whether eosinophils, like T cells,⁸⁻¹⁰ show differences in cytokine profiles in the different disorders. Second, we aimed to investigate the hypothesis that eosinophils play a central role in the pathogenesis of corneal disease in ocular allergy. We therefore examined for differences in eosinophil numbers (either absolute numbers or as a percentage of total leukocyte numbers) or in the degree of eosinophil activation or eosinophil cytokine production profile between the chronic ocular allergies that affect the cornea (AKC and VKC) and those that do not (GPC). Cytokines were chosen that are known to be associated with eosinophils in other tissues.

METHODS

Subjects

Conjunctival biopsy specimens were obtained from subjects with active VKC ($n = 10$, 2 women, mean age $\pm$ SEM = 21.1 ± 2.0 years), GPC ($n = 10$, 3 women, mean age $\pm$ SEM = 41.8 ± 6.3 years), and AKC ($n = 10$, 3 women, mean age $\pm$ SEM = 27.9 ± 2.3 years). Patients with GPC had biopsies performed before the onset of treatment, and patients with VKC and AKC had received no topical steroids for 3 months and no topical cromones for 1 week. The diagnoses were made clinically.¹⁻⁵ All patients with AKC also had atopic dermatitis, and all patients with VKC or AKC had current or previous corneal involvement. To ensure no confusion regarding the distinction between cases of VKC and AKC, all patients in the VKC group had a disease onset before 10 years of age, and all those in the AKC group had a disease onset after 18 years of age. The age of patients with VKC was higher than might be expected¹ because ethical approval of this study demanded patients older than 16 years (to ensure cooperation with tissue harvesting under local anaesthetic). As controls, biopsy specimens were obtained from 10 normal, non-age-matched, nonatopic patients (6 women, mean age $\pm$ SEM = 55.2 ± 6.4 years) undergoing squint corrections or cataract surgery. Patients with a history or signs of ocular inflammatory disease, external ocular disease, systemic inflammatory disorders, or contact lens wear were not used as control subjects. Informed consent was obtained from all participants, and all procedures were approved by the Ethics Committee of Moorfields Eye Hospital.

TABLE I. List of primary mAbs used in immunohistochemistry

mAb	Specificity	Source
Antibodies to cell surface antigens		
CD4	T-cell subsets and some eosinophils	Dako Ltd, High Wycombe, UK
CD8	T-cell subset	Dako
CD20	B cells (not plasma cells)	Dako
NPE	Neutrophils (elastase)	Dako
EG-2	Eosinophils (cationic protein)	Sera-Lab, Sussex, UK
AA-1	Mast cells	Dako
CD68	Macrophages, monocytes	Dako
CD25	IL-2R	Dako
HLA-DR	MHC class II molecules	Dako
CD54	ICAM-1	Dako
Antibodies to cytokines		
IL-3	Interleukin-3	Genzyme Diagnostics
IL-4	Interleukin-4	Genzyme Diagnostics
IL-5	Interleukin-5	Genzyme Diagnostics
IL-6	Interleukin-6	Genzyme Diagnostics
GM-CSF	Granulocyte-monocyte colony stimulating factor	Genzyme Diagnostics
RANTES	Regulated upon activation, normal T cells expressed and secreted	R&D systems, Abingdon, UK
IL-8	Interleukin-8	Genzyme Diagnostics
TGF- β	Transforming growth factor- β	Genzyme Diagnostics
TNF- α	Tumor necrosis factor- α	Genzyme Diagnostics

Biopsies

The upper tarsal conjunctiva was anaesthetized with 1% amethocaine drops and 2% lignocaine with adrenaline infiltration. In the control subjects, biopsy specimens were harvested before the commencement of surgery. By using a 3-mm trephine, specimens were taken from the central third of the superior tarsal conjunctiva, which is the site of maximum inflammatory conjunctival involvement in these disorders.

The biopsy specimens were immediately placed in ice-cooled acetone containing the protease inhibitors iodoacetamide (20 mmol/L) and phenylmethyl sulfonyl fluoride (2 mmol/L) and stored overnight at -20°C . The specimens were then processed for, and embedded in, glycol methacrylate resin (TAAB Laboratories, Aldermaston, UK).²⁵ The blocks were stored at -20°C .

Immunohistochemistry

An ultramicrotome was used to cut 2- μm sections, which were floated on 0.2% ammonia water, placed on amino-propyl triethoxy silane (Sigma Chemicals, Poole, UK) coated slides, and allowed to air dry. An indirect streptavidin-biotin-peroxidase complex technique was used. Endogenous peroxidase was inhibited with a solution of 0.3% hydrogen peroxide in 0.1% sodium azide (Sigma chemicals, Poole, UK) for 10 minutes followed by 2 5-minute washes in PBS (Sigma chemicals) and incubation for 30 minutes with 10% normal rabbit serum (Dako Ltd, High Wycombe, UK) to prevent nonspecific binding of mAb. The sections were then incubated overnight with 1 of a panel of mouse anti-human antibodies (IgG1) at appropriate dilutions (Table I).

After washing in PBS, biotinylated anti-mouse antibody (Dako Ltd) was applied to the sections for 2 hours. After a further wash in PBS, the slides were incubated with streptavidin-peroxidase (Dako Ltd) for 2 hours, again washed in PBS, and developed for 25 minutes with a solution of amino-ethyl carbazole (AEC) (Sigma Chemicals) and peroxide. For cell counts, the slides were then rinsed in tap water, counterstained with Mayer's hematoxylin, and mounted with Glycergel (Dako Ltd). For the localization of antigens to eosinophils, the slides were washed with PBS, and the immunohistochemistry process was repeated with mouse anti-human eosinophil cationic

protein antibody as the primary antibody, biotinylated anti-mouse antibody (Dako Ltd) as the secondary antibody, and diaminobenzamine (DAB) (Vector Laboratories, Peterborough, UK) instead of AEC as chromagen. Once again, incubation with the primary antibody was preceded by exposure of sections to normal rabbit serum to minimize nonspecific binding. Immunohistochemistry was performed on tonsil sections with and without primary antibodies against CD4 for positive and negative controls, respectively, and on conjunctival sections with an unrelated mouse mAb of the same isotype (anti-cytomegalovirus, Dako Ltd) (Fig 1).

Hematoxylin and eosin staining was used for morphologic definition and orientation of the sections and to identify plasma cells.

Cell counts and statistical analysis

After immunohistochemistry, cells staining positively for cell identification markers (eg, CD20 for B cells), cytokines, and eosinophil activation markers were identified by a red deposition of AEC reaction product (Fig 1). Plasma cells were identified by their characteristic morphology on hematoxylin and eosin sections (large amounts of agranular cytoplasm with a pale staining perinuclear area and peripherally clumped chromatin in an eccentric nucleus). Eosinophils were identified by the black-staining DAB reaction product, and eosinophils were stained for cytokines or cell surface antigens by a brown or combined red-black color because of the combination of a positive reaction to AEC and DAB (Fig 2).

Grading of the degree of staining and cell counts were performed by a masked observer with an Olympus BH2 microscope by using a 1-mm² eyepiece graticule at $\times 400$ magnification. For each section, 150 cells or fewer were counted in at least 3 adjacent fields (as limited by size of specimen) and expressed as mean counts per square millimeter of substantia propria. Individual leukocyte counts were summed for a total leukocyte count to allow calculation of the percentage of leukocytes that were eosinophils. For eosinophil double-staining, the results were expressed as the percentage of samples exhibiting any double-staining, and the percentage of eosinophils in each sample that were double-stained was calculated.

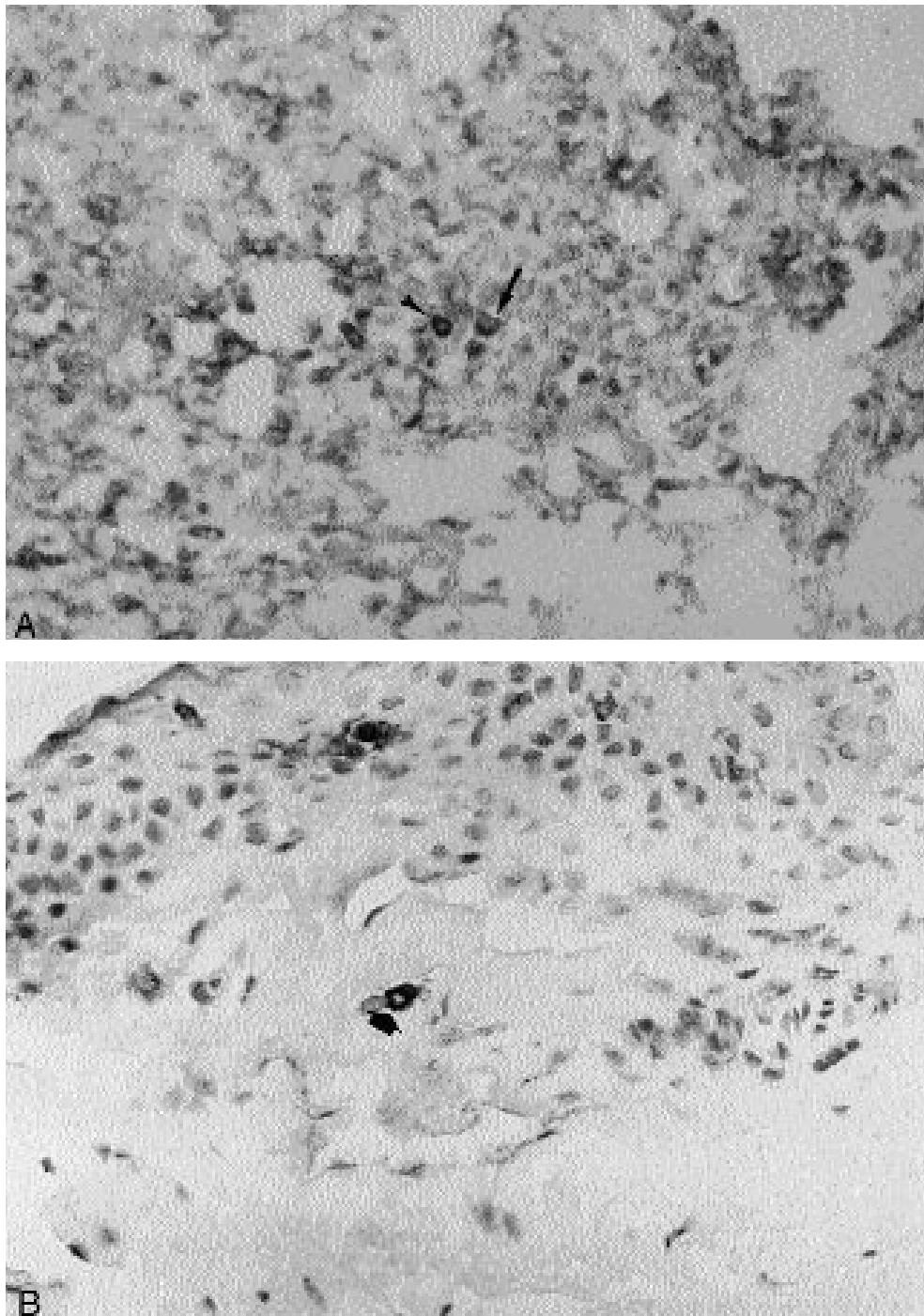


FIG 2. A, Example of DAB single-positive cell and AEC-DAB double-positive cell. An eosinophil stains black (ECP immunoreactivity, DAB chromagen, *black arrow*); an eosinophil expressing IL-5 (AEC chromagen) shows a combined red and black stain (*arrowhead*) (6- μ m section). Example of AEC-DAB double-positive cell. **B,** An eosinophil expressing ICAM-1 (2- μ m section) ($\times 100$, hematoxylin counterstain).

A nonparametric statistical test (Mann-Whitney U test, two-tailed) was used to analyze the differences in cell numbers and percentages between the disease groups, and Fisher's exact test (two-tailed) was used to analyze differences in the numbers of samples. A *P* value of less than .05 was taken as statistically significant.

RESULTS

Cell counts

Cell counts are listed in Table II. There were very few eosinophils in the normal human tarsal conjunctiva, but

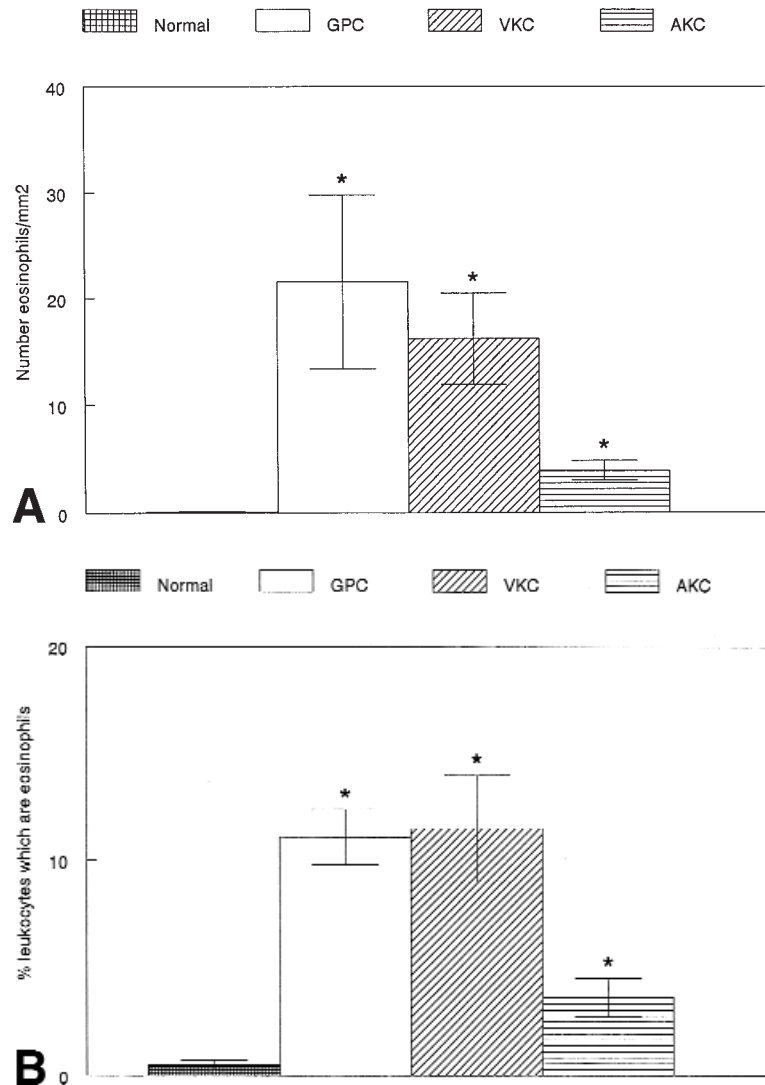


FIG 3. Numbers of eosinophils in substantia propria in normal and allergic conjunctiva. **A**, Numbers (mean, SEM) of eosinophils per square millimeter substantia propria. **B**, Eosinophils as percentage of total number of infiltrating leukocytes. *Significant differences from normal, $P < .05$.

there was a marked eosinophil infiltration in all the diseased tissues examined, with significantly greater numbers of eosinophils in all the disorders than in normal subjects (Fig 3). The eosinophil infiltrate was more dense in GPC (mean $\pm$ SEM = $21.6 \pm 8.2/\text{mm}^2$) and VKC ($16.2 \pm 4.3/\text{mm}^2$) than in AKC ($3.6 \pm 0.9/\text{mm}^2$), and these differences were statistically significant ($P = .002$ and $P = .009$, respectively). Eosinophils constituted a greater percentage of infiltrating leukocytes in the allergic tissue (GPC, $11.1 \pm 1.3/\text{mm}^2$, $P = .002$; VKC, $11.5 \pm 2.5/\text{mm}^2$; $P = .0003$, and AKC, $3.6 \pm 0.9/\text{mm}^2$, $P = .007$) than in normal tissue ($0.5 \pm 0.2/\text{mm}^2$). Eosinophils accounted for a greater percentage of conjunctival leukocytes in GPC ($P = .001$) and VKC ($P = 0.01$) than in AKC.

Eosinophil cell surface antigens

The results of double-staining of eosinophils for cell surface antigens is shown in Fig 4. The few eosinophils that were present in normal conjunctiva did not express any of the antigens investigated.

There was greater expression of all the eosinophil cell surface antigens examined in AKC and VKC than in GPC. The frequency of samples containing eosinophils expressing these antigens was more than twice as high in VKC and AKC compared with GPC for all markers (CD4: 50% VKC, 60% AKC, 20% GPC; HLA-DR: 70% VKC, 80% AKC, 30% GPC; intercellular adhesion molecule [ICAM]-I: 80% VKC, 70% AKC, 30% GPC; and IL-2R: 60% VKC, 60% AKC, 0% GPC). The percent-

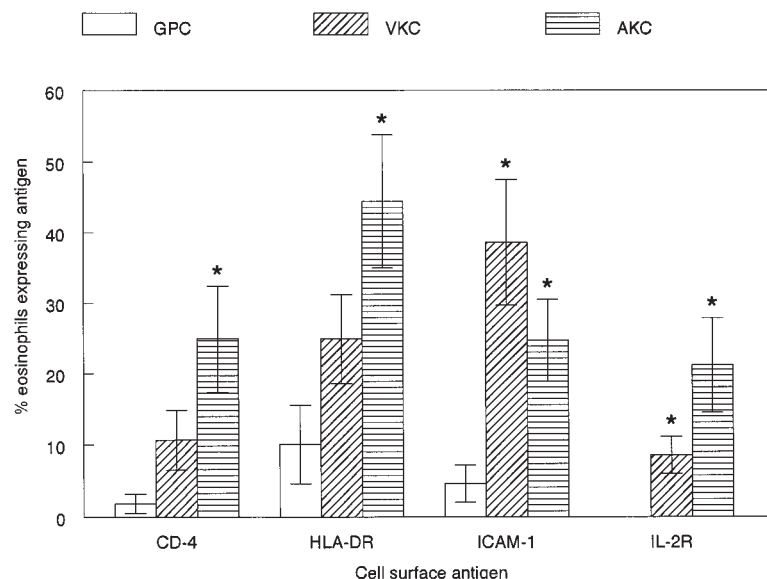


FIG 4. Conjunctival eosinophil expression of CD4, HLA-DR, ICAM-1, and IL-2R by immunohistochemistry. Mean (SEM) percentage of conjunctival eosinophils expressing antigens. *Significant inter-disease differences, $P < .05$.

ages of tissue eosinophils staining for surface antigen in each sample were also higher in VKC and AKC than GPC for all cell surface antigens (all values are mean $\pm$ SEM; CD4: $10.7\% \pm 4.2\%$ VKC, $24.9\% \pm 7.5\%$ AKC, $1.8\% \pm 1.3\%$ GPC; HLA-DR: $24.9\% \pm 6.3\%$ VKC, $44.4\% \pm 9.4\%$ AKC, $10.1\% \pm 5.5\%$ GPC; ICAM-1: $38.6\% \pm 8.9\%$ VKC, $24.7\% \pm 5.8\%$ AKC, $4.6\% \pm 2.6\%$ GPC; and IL-2R: $8.6\% \pm 2.6\%$ VKC, $21.2\% \pm 6.7\%$ AKC, 0% GPC). This reached statistical significance in AKC for CD4 ($P = .03$), HLA-DR ($P = .01$), ICAM-1 ($P = .01$), and IL-2R ($P = .000$) and in VKC for ICAM-1 ($P = .07$) and IL-2R ($P = .000$). IL-2R staining was seen only on eosinophils in VKC and AKC. There were no significant differences between AKC and VKC.

In the pathologic specimens in which CD4⁺ eosinophils were present, they constituted $20.3\% \pm 4.2\%$ (range, 2.9% to 44.4%) of all CD4⁺ cells. This did not vary significantly between the different clinical disorders ($24.1\% \pm 16\%$ GPC, $17.1\% \pm 7.2\%$ VKC, and $21.7\% \pm 5.9\%$ AKC).

Cytokine localization to eosinophils

Results for each disorder are given as mean $\pm$ SEM percentage of eosinophils with positive cytokine staining (percent eosinophils) and percentage samples containing eosinophils with positive cytokine staining (percent of samples) (Fig 5). There were very few eosinophils in normal conjunctival samples, but in none of these could cytokine colocalization be demonstrated. A number of cytokines localized to eosinophils in all the allergic eye disorders to a similar degree. These were RANTES (VKC $9.7\% \pm 5.0\%$ eosinophils, 30% samples; AKC $11.6\% \pm 6.1\%$ eosinophils, 30% samples; and GPC $1.3\% \pm 1.3\%$ eosinophils, 10% samples), transforming growth

factor (TGF)- β (VKC $18.7\% \pm 8.5\%$ eosinophils, 40% samples; AKC $21.8\% \pm 7.7\%$ eosinophils, 50% samples; and GPC $11.7\% \pm 6.6\%$ eosinophils, 30% samples), and TNF- α (VKC $28.7\% \pm 8.8\%$ eosinophils, 70% samples; AKC $22.4\% \pm 7.7\%$ eosinophils, 50% samples; and GPC $18.0\% \pm 7.4\%$ eosinophils, 60% samples). Certain cytokines were localized to eosinophils only in certain disorders. Eosinophil IL-3 was observed in VKC ($22.5\% \pm 6.0\%$ eosinophils, 70% samples) and only minimally in AKC ($1.5\% \pm 1.5\%$ eosinophils, $P = .006$; 10% samples, $P = .02$) and not in GPC ($P = .0021$). Eosinophil IL-5 occurred in GPC ($7.5\% \pm 3.6\%$ eosinophils, 40% samples) and VKC ($18.5\% \pm 5.5\%$ eosinophils, 60% samples) only to a similar extent.

Eosinophil IL-6 was greater in VKC ($17.0\% \pm 7.3\%$ eosinophils, 50% samples) than in GPC ($0.42\% \pm 0.42\%$ eosinophils, $P = 0.04$, 10% samples) and also greater (but not reaching statistical significance) than AKC ($5.3\% \pm 3.9\%$ eosinophils, 20% samples). IL-4 colocalization was seen in all the disease groups but was significantly greater in AKC ($41.1\% \pm 9.3\%$ eosinophils, 90% samples) than GPC ($4.3\% \pm 2.5\%$ eosinophils, $P = .001$; 30% samples, $P = .02$) and VKC ($5.7\% \pm 2.5\%$ eosinophils, $P = .002$; 40% samples $P = .04$). GM-CSF colocalization was much greater in both AKC ($29.8\% \pm 8.4\%$ eosinophils, $P = .01$; 60% samples, $P = .04$) and VKC ($21.8\% \pm 6.9\%$ eosinophils, $P = .01$; 60% samples, $P = .04$) than GPC ($1.5\% \pm 1.5\%$ eosinophils, 10% samples). IL-8 colocalization was greater in AKC ($36.6\% \pm 10.7\%$ eosinophils, 70% samples) than VKC ($2.4\% \pm 1.6\%$ eosinophils, $P = .009$; 20% samples, $P = .06$) and GPC ($10.7\% \pm 7.2\%$ eosinophils, $P = .05$; 20% samples, $P = .06$).

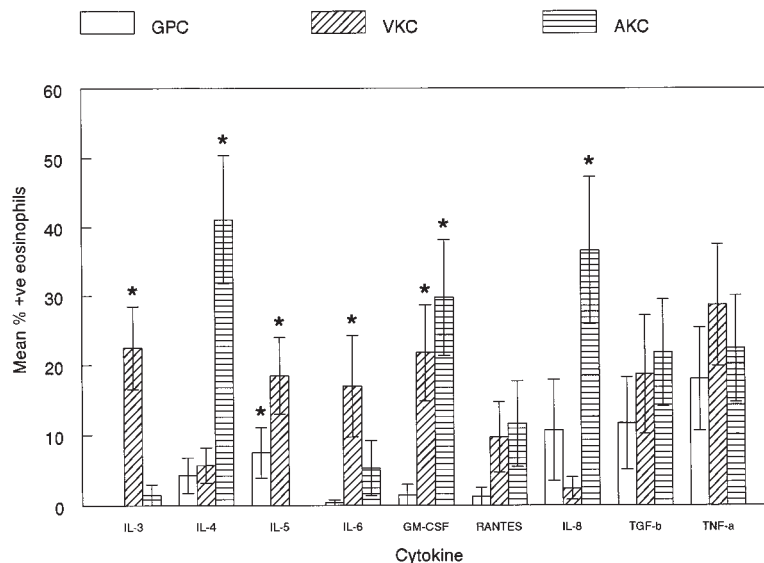


FIG 5. Conjunctival eosinophil expression of cytokines by immunohistochemistry. Mean (SEM) percentage of conjunctival eosinophils that stain for cytokine. *Significant inter-disease differences, $P < .05$.

DISCUSSION

Tissue for analysis was obtained from the superior tarsal conjunctiva, which is the area of maximal clinical inflammation in GPC, VKC, and in our experience, AKC. Although some authors quote the inferior tarsal conjunctiva as the site of maximal clinical involvement,²⁶ this is not the case for UK^{7,27} and some other groups.^{28,29} The use of glycolmethacrylate resin allowed ultrathin (2 μ m) sectioning, which permits very accurate cell counting. We chose to use mAbs against specific eosinophil granule proteins for eosinophil counts and localization of antigens to eosinophils, rather than identifying eosinophils by their characteristic morphology. This is because this morphology may be lost or obscured in degranulated or degranulating cells,³⁰ leading to underidentification of eosinophils.

A number of lines of evidence have suggested that eosinophils are central to the pathogenesis of allergic corneal damage.^{31,32} The data from this study did not show increased numbers of eosinophils (expressed either as absolute numbers or as a percentage of infiltrating leukocytes) in the disorders with keratopathy. Although this does not apparently support a direct role of eosinophils in corneal disease, we also investigated whether there is any link between corneal involvement and differences in eosinophil function by assessing cell surface antigen and cytokine expression. Certain antigens are induced or upregulated on the surface of cytokine-stimulated eosinophils or eosinophils from patients with atopic disease or hypereosinophilia.³³⁻³⁸ The results showed that there was increased expression of such antigens (CD4, HLA-DR, ICAM-1, and IL-2R) in those disorders that affect the cornea. This suggests that there is a greater

degree of eosinophil activation in those disorders affecting the cornea than in those that do not and that it may be the level of eosinophil activation, rather than eosinophil numbers, that is important in the development of corneal disease through greater release of epitheliotoxic mediators and cytokines from activated eosinophils.

The conventional view of the eosinophil as a simple effector leukocyte in allergic inflammation is no longer tenable.³⁹ Cytokine mRNA and product may be expressed constitutively in peripheral blood eosinophils from normal humans (IL-6, IL-8, RANTES, TGF- α , and TNF- α)⁴⁰⁻⁴⁵ or from patients with hypereosinophilia (IL-5, IL-6, TNF- α , TGF- α , TGF- β , and macrophage inhibitory protein-1 α).^{41,43,46-50} In atopic disease blood eosinophils express IL-5 and increased levels of IL-8 and can be stimulated to synthesize IL-3.^{42,51,52}

Tissue eosinophils are producing cytokines in disease. In nasal allergy and polyposis, IL-4, IL-5, TNF- α , TGF- α , TGF- β , and macrophage inhibitory protein-1 α mRNA or protein have been colocalized to eosinophils.^{43,53-59} In asthma, eosinophils in bronchoalveolar lavage contain IL-5 mRNA,⁶⁰ and mucosal eosinophils contain IL-5 protein⁶¹ and IL-4 mRNA.⁵⁵ In atopic dermatitis, eosinophils contain IL-5 mRNA.⁵¹ Other disorders in which eosinophil cytokines have been identified include coeliac disease (IL-5),⁴⁶ eosinophilic cystitis (IL-5),⁴⁷ dermatitis herpetiformis (IL-5),⁶² necrotizing enterocolitis (TNF- α),⁶³ colonic adenocarcinoma and oral squamous cell carcinoma (TGF α),⁴⁸ Hodgkin's disease (TGF- β),⁶⁴ and pemphigoid (IL-10 and IFN- γ).⁶⁵

Thus eosinophils can potentially produce a wide range of cytokines. However, we do not yet know the pattern and range of tissue eosinophil cytokine production in any one

TABLE II. Leukocyte cell counts in the upper tarsal conjunctiva in subjects with chronic allergic eye diseases and control subjects

	Normal	GPC	VKC	AKC
Eosinophils	0.1 ± 0.06	21.6 ± 8.2 (<i>P</i> = .0002)	16.2 ± 4.3 (<i>P</i> = .0001)	3.6 ± 0.9 (<i>P</i> = .0003)
Neutrophils	2.9 ± 0.4	31.0 ± 10.5 (<i>P</i> = .001)	20.5 ± 7.3 (<i>P</i> = .0002)	70.1 ± 13.6 (<i>P</i> = .0002)
CD4 ⁺ cells	3.3 ± 0.5	16.8 ± 4.3 (<i>P</i> = .0006)	18.1 ± 4.4 (<i>P</i> = .0002)	11.7 ± 2.6 (<i>P</i> = .02)
CD8 ⁺ cells	5.5 ± 1.0	25.5 ± 8.9 (<i>P</i> = .003)	14.7 ± 2.8 (<i>P</i> = .01)	6.2 ± 1.6
CD4/CD8	0.7 ± 0.1	0.8 ± 0.1	1.6 ± 0.4	2.4 ± 0.8 (<i>P</i> = .01)
B cells	6.6 ± 1.1	39.8 ± 10.5 (<i>P</i> = .002)	31.1 ± 5.7 (<i>P</i> = .0002)	15.2 ± 1.9 (<i>P</i> = .002)
Plasma cells	1.2 ± 0.5	9.3 ± 2.4 (<i>P</i> = .005)	7.4 ± 0.8 (<i>P</i> = .003)	5.7 ± 1.0 (<i>P</i> = .001)
Macrophages	3.8 ± 0.5	7.5 ± 1.3 (<i>P</i> = .02)	13.7 ± 2.5 (<i>P</i> = .0008)	10.2 ± 1.3 (<i>P</i> = .0005)
Mast cells	1.3 ± 0.3	15.7 ± 4.6 (<i>P</i> = .0002)	8.2 ± 1.6 (<i>P</i> = .0002)	8.6 ± 2.1 (<i>P</i> = .0002)
Total leukocytes	25.9 ± 1.8	169.4 ± 46.5 (<i>P</i> = .002)	129.9 ± 12.6 (<i>P</i> = .002)	127.1 ± 2.1 (<i>P</i> = .002)

Results expressed as mean ± SEM cell count/mm² substantia propria. Probability (*P*) values shown for significant differences in cell counts between allergic disease and normal.

disorder or whether the spectrum of eosinophil cytokines differs between different groups of disorders (eg, atopic vs neoplastic), between different disorders with broadly similar pathophysiology (eg, asthma vs atopic dermatitis), or between individuals with the same diagnosis (eg, with disease severity). The relationship between eosinophil cytokine production and that from T cells, mast cells, and other sources is also undefined.

We have been able to use immunohistochemistry for the study of eosinophil cytokines because, like mast cells, they contain significant amounts of stored cytokines. In comparison, T-cell work relies mainly on detection of mRNA by in situ hybridization because T-cell cytokines are released almost immediately.¹¹ Our work suggests that eosinophils are an important source of cytokines in serious ocular allergic disease, and it shows that the spectrum of cytokines localizing to eosinophils differs in the different disorders, although we acknowledge that the technique does not measure cytokine release or prove cytokine synthesis, which requires in situ hybridization (work in progress).

The conclusion from this study is that it is not the extent of eosinophil infiltration per se but the extent of eosinophil activation and the pattern of cytokine expression that is indicative of disease severity and clinical variations such as involvement of the cornea. Intracellular cytokine expression does not necessarily imply cytokine secretion, and unless cells are activated and degranulating, cytokine will not be released in large quantities. Thus although eosinophils are present in high numbers in GPC, their level of activation is lower (suggesting cytokine might not be released), and their pattern of cytokine expression (mainly IL-5) is different from the other clinical groups. The main action of IL-5 is in enhancing eosinophil survival and the protection of these cells from apoptosis, but it need not be involved in the mechanisms of tissue destruction or activation of other cell types.^{66,67} Having one predominant cytokine involved also suggests an obvious target for immunotherapy.

In contrast, the enhanced activation of eosinophils in AKC and VKC suggests that these cells are likely to release their cytokines and could play a role in the perpetuation of the disease. In addition, their cytokine profiles are different. In

VKC there is expression of IL-3, IL-5, IL-6, and GM-CSF. These cytokines are proinflammatory and can be chemoattractants for other cell types (eg, mast cells and B cells), and this would in turn lead to other cytokines being produced within the tissue.⁶⁸⁻⁷⁰ In AKC there is expression of IL-4, IL-8, and GM-CSF. IL-4 is not only a B-cell stimulator but can also influence the development of T cells toward a T_{H2}-like phenotype.⁷¹ Although mast cells are known to synthesize and secrete IL-4 in ocular allergy,⁷² the role of eosinophils in producing IL-4 in AKC has not previously been demonstrated. IL-8 is a potent neutrophil chemoattractant,⁷³ and therefore in AKC the activated eosinophils are likely to contribute to a wider variety of pathways, which could be involved in the chronicity and corneal involvement observed. It remains to be seen whether the common cytokine detected in VKC and AKC eosinophils (GM-CSF) is the one that dictates whether corneal involvement occurs.

Although other cells (T cells and mast cells) are present in the inflammatory infiltrate, the results in this study support an important role for eosinophils in the pathogenesis of disease. Previous work has shown functionally distinct T-cell subsets in the different chronic ocular allergic diseases.¹⁰ It may be that differences in eosinophil cytokine production are secondary to these differences in T cells (or other cells such as mast cells) or a primary phenomenon that could itself influence T-cell behavior. These eosinophil differences may be reflections of similar differences in the circulating eosinophil pool or may be influenced by variations in local allergenic exposure, by the age at allergen exposure or by disease chronicity. It may also be possible to relate such differences to disease characteristics other than corneal disease, such as the degree of conjunctival cicatrization (much greater in AKC than VKC). We note that none of the cytokines were positive in all cases in any disease group. We believe this may be related to interpatient differences in clinical characteristics.

The conjunctiva provides a particularly useful location for the study of eosinophil function in the allergic process. Eosinophil infiltration is characteristic in ocular allergy, and mucosal tissue is far more easily accessible for biopsy and direct observation than that of the lung or even the

nose. Visualization of the mucosal surface has also allowed a more accurate subclassification of allergic disease than in almost any other system. In past years, findings from other allergic conditions, such as asthma, have been extrapolated to the conjunctiva to explain the pathophysiology and to suggest new therapeutic possibilities.⁶⁷ It may be more expedient to extrapolate in the opposite direction for the reasons stated above, although we do not yet know the relevance of our findings to atopic disease in general. Potentially the classification of ocular allergic disease has parallels with that in other atopic disease. The effects of new antiallergic drugs can be more easily assessed in the eye by observation and biopsy of the ocular mucosa than they can in the lung, in which direct mucosal assessment is often impractical or unsafe.

The function of the eosinophil is likely to be central to the understanding of ocular and other allergic diseases. We have begun to characterize some eosinophil mechanisms in detail. Similar studies in other tissues will allow comparison with asthma, rhinitis, and atopic dermatitis.

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