The immunopathogenesis and treatment of chronic allergic eye disease

A thesis submitted for the degree of Doctor of Medicine

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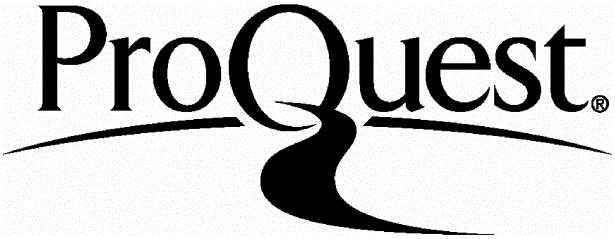

Abstract

The pathogenesis of the potentially blinding chronic ocular allergies is poorly understood, and current therapy relies on topical steroids which have vision-reducing side-effects. This thesis aimed to elucidate this pathogenesis, particularly of sight-damaging corneal involvement, and to evaluate therapeutic topical cyclosporin A in atopic keratoconjunctivitis (AKC). The techniques employed were conjunctival biopsy, immunohistochemistry, *in situ* hybridisation and a randomised, placebo-controlled clinical trial.

An initial examination of normal conjunctival leukocytes showed the relative frequencies of different leukocytes and that lymphocytes had features of mucosal-associated lymphoid tissue but organised conjunctival-associated lymphoid tissue was infrequent.

An examination of T cells, eosinophils and epithelial cells was conducted. Vernal keratoconjunctivitis and giant papillary conjunctivitis had predominantly Th2-like cells (resembling asthma) but AKC resembled atopic dermatitis with a shift towards a Th1-pattern. There was greater expression of eosinophil surface antigens (and possibly activation) associated with corneal disease and variations in patterns of eosinophil-cytokine localisation occurred between individual disorders. Epithelial cell expression of pro-inflammatory surface antigens was particularly upregulated in disorders with keratopathy and there were different patterns of cytokine co-localisation in the various diseases.

A trial of topical cyclosporin A demonstrated clinical improvement and reduced steroid requirement in AKC associated with reductions in leukocyte numbers and T cell cytokine expression not seen with placebo. The drops were difficult to tolerate.

These results show a complex pathogenesis of cellular and cytokine interactions underlying chronic allergic eye disease with certain parallels to systemic allergic disorders. These results may allow future therapeutic developments with greater specificity and fewer side-effects than topical steroids.

Declaration and conjoint statement

All the work undertaken in this thesis is my own, except:

1. Recruitment and recording of clinical details: This was performed by Miss Annette Bacon (whilst a registrar in Ophthalmology, Moorfields Eye Hospital) for the following patients: the 20 patients in whom normal conjunctiva only was examined (Chapter 3, Appendix 1a); the 8 patients with GPC, 8 with VKC and 8 with AKC and the 10 normal controls in whom in situ hybridisation was performed (Chapter 4, Appendices 1b and 2). For all other patients recruitment and recording of clinical details was performed by me.

2. Examination of the normal conjunctival leukocytes (Chapter 3): Immunohistochemistry for the antigens CD3, CD45Ro, CD4, CD8, CD25, CD68, HLA-DR, CD20, CD57 and eosinophil cationic protein was performed by Daniella Metz (research student, Institute of Ophthalmology); immunohistochemistry for the antigens AA-1, TCR-γδ, HML-1 and neutrophil elastase was performed by me.

3. All in situ hybridisation (Chapter 4) was performed by Daniella Metz.

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My biggest debt of professional gratitude is to Professor Susan Lightman, my supervisor. She has guided and supported me without fail from my appointment at Moorfields Hospital to this day, is always available and approachable despite the huge demands on her time, and possesses uncannily accurate timing in providing firm direction or encouraging independence in her staff. My wholehearted thanks go to her.

Finally but most importantly, I dedicate this thesis to Aroon, my husband. Work is important in life but love gives it meaning. Thank you for giving me the sort of happiness that I probably don't deserve and certainly cannot express in words.

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Abbreviations

AD	atopic dermatitis
AKC	atopic keratoconjunctivitis
APC	antigen presenting cell
BAL	bronchoalveolar lavage
CALT	conjunctival-associated lymphoid tissue
CD	cluster differentiation
CLC	Charcot-Leyden crystal
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EpDRF	epithelial-derived relaxing factor(s)
EpDCF	epithelial-derived contracting factor(s)
EPO	eosinophil peroxidase
EPR	early phase reaction
FcεR	IgE receptor
FGF	fibroblast growth factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GPC	giant papillary conjunctivitis
HETE	hydroxyeicosatetraenoate
HLA	human leukocyte antigen
HML-1	human mucosal lymphocyte antigen-1
ICAM	intercellular adhesion molecule
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IFN	interferon
IGF	insulin-like growth factor
IL-	interleukin
IL-2R	interleukin-2 receptor
LCA	leukocyte common antigen
LCF	lymphocyte chemoattractant factor
LFA	leukocyte function antigen
LIF	lymphocyte inhibitory factor
LPL	lamina propria lymphocyte
LPR	late phase reaction
LT	leukotriene
MALT	mucosal-associated lymphoid tissue
MBP	major basic protein

MC	mast cell
MCt	tryptase-only containing mast cell
MCtc	tryptase and chymase-containing mast cell
M-cell	microfold cell
M-CSF	macrophage colony stimulating factor
MCP	monocyte chemotactic protein
MHC	major histocompatibility
MIP	macrophage inhibitory protein
NK	natural killer
PAF	platelet activating factor
PDGF	platelet-derived growth factor
PECAM	platelet endothelial cell adhesion molecule
PG	prostaglandin
PMFA	protein migrating faster than albumin
RANTES	regulated upon activation, normal T cells expressed and secreted
SAC	seasonal allergic conjunctivitis
SCF	stem cell factor
SiLewX	Sialyl-Lewis X
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumour necrosis factor
Tx	thromboxane
VCAM	vascular cell adhesion molecule
VKC	vernal keratoconjunctivitis
VLA	very late antigen

Summary

Ocular allergy is common and the more serious forms, vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) are potentially blinding, especially in certain geographical areas, such as Africa and the Middle East. The aims of the studies described were to examine the pathogenesis of serious chronic allergic eye disease, particularly the visually-threatening complication of corneal damage, (using as controls both normal subjects and subjects with giant papillary conjunctivitis [GPC], a chronic ocular allergy similar to VKC which does not cause corneal damage), and to evaluate the effects of cyclosporin A (CsA) in drop form in AKC. The studies can be divided into several parts.

Characterisation of the normal conjunctival leukocyte population
Lymphoid cells are present in normal mucosal tissue as scattered cells or organised into mucosal-associated lymphoid tissue (MALT). Knowledge of the non-pathological conjunctival lymphoid tissue is a vital basis for the further study of ocular surface inflammatory disorders such as ocular allergy. Therefore, immunohistochemistry (IHC) was used to examine the leukocyte population of tarsal and bulbar conjunctiva from normal patients with no ocular or systemic inflammatory disease.

$CD3^+$ T cells were the most frequently occurring, and macrophages the second most frequently occurring, conjunctival cell type and were seen in the epithelium and substantia propria. There were greater numbers of leukocytes in the bulbar than in the tarsal area and this reached statistical significance for $CD3^+$ T cells and $CD57^+$ natural killer (NK) cells. In both bulbar and tarsal conjunctiva, B cells and neutrophils were seen in the epithelium and substantia propria, but plasma cells, NK cells and mast cells were present only in the substantia propria. No eosinophils were seen. $CD8^+$ cells outnumbered $CD4^+$ cells in the epithelium (CD4:CD8 0.3) but this was reversed in the substantia propria (CD4:CD8 1.3 tarsal, 2.0 bulbar). Most epithelial T cells and half the stromal T cells were $CD45Ro^+$. Interleukin (IL)-2R (CD25) staining was infrequent in the tarsal but not the bulbar area. The greater number and activation of T cells in the bulbar region may relate to greater antigen exposure. HLA-DR^+ cells were mainly macrophages, but also included dendritic cells in the epithelium and a few epithelial cells. The conjunctival lymphocytes do have certain features of MALT cells, apart from mucosal recirculation, homing and promotion of sIgA production. Conjunctival intraepithelial lymphocytes (IEL), like gut IEL, are predominantly $CD8^+$, are found in the basal epithelium, are human mucosal lymphocyte antigen (HML)-1^+ and have very high expression of CD45Ro.

Substantia propria lymphocytes have more equal CD4 and CD8 numbers and frequently express CD45Ro. We found only one example of organised conjunctival lymphoid tissue in our study and suggest that the presence of conjunctival-associated lymphoid tissue (CALT) in humans is not universal.

T cell cytokines in chronic allergic eye disease

The pathophysiology of chronic allergic eye disease cannot be explained by type I hypersensitivity alone and T cell-mediated inflammation is strongly implicated. Previous studies suggested that T helper (Th)2-like T cells play an important role in one form of chronic allergic eye disease (VKC). This study examined the cytokine profile of T cells in the different clinical groups of chronic allergic eye disease (VKC, AKC and GPC) and normal controls.

In situ hybridisation (ISH) was used to identify cytokine mRNA and two-colour IHC used to demonstrate cytokine immunoreactivity localising to T cells in the conjunctiva.

Allergic tissue expressed increased levels of mRNA for IL-3, IL-4 and IL-5 compared with normal. There was significantly greater IL-2 mRNA expression in AKC than in VKC ($p=0.004$) and GPC ($p=0.02$). Immunoreactivity for T cell IL-5 was present more frequently in VKC ($p=0.004$), GPC ($p=0.02$) and AKC ($p=0.04$) than in normal controls. However, T cell IFN-γ protein expression was greater in AKC than in VKC ($p=0.01$), GPC ($p=0.01$) and normal ($p=0.005$).

These results show a Th2-like T cell cytokine array in VKC and GPC whereas in AKC there is a shift in cytokine profile towards a more Th1-like pattern, potentially due to differences in chronicity of the disorders. These important functional T cell variations in chronic allergic eye conditions are likely to be important in understanding differences in clinical characteristics and therapeutic responses. These differences parallel those between asthma and early atopic dermatitis (AD) compared with established AD.

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

The pathophysiology of chronic ocular allergic disease is still not well understood. An eosinophil infiltrate is characteristic and eosinophil activity can be related to disease severity and to keratopathy (corneal damage), the most serious complication. Recently, eosinophils have been shown to be capable of cytokine production, particularly in allergic disease, although the disease-specific cytokine spectrum of tissue eosinophils is unknown. The aim of this study was 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. Ultra-thin (2μm) sections of conjunctiva were examined by tissue staining and by one- and two-colour IHC.

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

Chronic ocular allergic disorders affecting the cornea are distinguished from disorders which do not by greater expression of eosinophil surface antigens (which may imply greater cell activation) and differences in cytokine localisation 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.

The role of conjunctival epithelial cells in chronic ocular allergic disease

Recent evidence suggests that mucosal epithelial cells are capable of actively participating in immune reactions via expression of surface antigens, such as adhesion molecules, and synthesis of cytokines. This appears to be important in the pathophysiology of non-ocular allergic disorders. The aim of this study was to compare the expression of HLA-DR, ICAM-1 and pro-allergic cytokines in conjunctival epithelial cells in the different chronic ocular allergic disorders with each other and with normal subjects.

Conjunctiva from normal patients and patients with VKC, AKC and GPC was examined by IHC. Epithelial cell staining for surface antigens and cytokines was graded using a four-point scale based on the percentage of epithelial cells staining positive.

There was no expression of ICAM-1 or HLA-DR in the normal conjunctival epithelial cells, but both antigens were induced on conjunctival epithelial cells in the allergic tissue, and there was greater expression in AKC and VKC compared with GPC. Cytokines IL-6, IL-8, RANTES and TNF-α all localised to normal conjunctival epithelial cells. RANTES was upregulated in all the allergic disorders and IL-8 was upregulated in GPC. IL-3 and GM-CSF were not expressed in normal conjunctival epithelial cells. GM-CSF was expressed in all disorders and there was greater expression in AKC compared with GPC and VKC. IL-3 was expressed only in AKC and VKC epithelial cells.

These results suggest that conjunctival epithelial cells play an important pro-inflammatory role in chronic ocular allergic diseases; ICAM-1 may allow epithelial cells to recruit, retain and locally concentrate leukocytes; the presence of HLA-DR raises the question of conjunctival epithelial cell antigen presentation. The epithelial cytokines which are upregulated are known to promote eosinophilic inflammation and are typical of allergic inflammation.

A randomised, placebo-controlled trial of topical CsA in steroid-dependent AKC

Subjects with AKC and VKC rely heavily on topical steroid therapy, which has sight-threatening side effects. CsA inhibits T cells and also has some inhibitory effects on eosinophils and mast cells. Topical CsA 2% produces clinical improvement and decreases steroid-dependence in VKC. The objective of this study was to investigate the therapeutic effect of topical CsA in steroid-dependent AKC, which might be different than that in VKC, given the differences in T cell subsets in the two disorders.

A prospective, randomised, double-masked, placebo-controlled trial was performed in 21 patients with steroid-dependent AKC. Patients used either topical CsA or vehicle four times daily for 3 months, in addition to their usual therapy, and the clinical response was used to taper or stop topical steroids where possible. The main outcome measures were steroid drop usage per week, ability to cease steroid use, scores for symptoms and clinical signs, drop side-effects and overall subjective rating of trial drop by patients and clinician.

CsA had a greater steroid-sparing effect than placebo. Nine of 12 CsA patients ceased steroids compared with 1 of 9 placebo patients ($p=0.01$), the final steroid use was lower in the CsA group (2.6 ± 1.4 vs. 27.7 ± 17.7, $p=0.005$) and the mean reduction in steroid use was greater for CsA (85.5 ± 14.7 vs. 13.9 ± 16.0, $p=0.005$). Clinical signs and symptom scores were reduced to a greater level for CsA. Significant side-effects

were lid skin maceration in 1 patient using CsA, and an allergic reaction in 1 placebo patient. Marked blurring of vision after drop instillation was common in both groups, but intense stinging more common in CsA patients (9/12 vs. 1/9, p=0.01), limiting frequency of drop use. The clinician rated the trial drops as good or excellent more frequently for CsA (11/12 vs. 0/9, p<0.0001).

It can be concluded that topical CsA is an effective and safe steroid-sparing agent in AKC and, despite difficulties in patient tolerance, also improves symptoms and signs.

The immunomodulatory effect of topical CsA in AKC

The clinical improvement and steroid-sparing effect of topical CsA in VKC is paralleled by changes in leukocyte numbers. Topical CsA appears also to be beneficial in AKC, despite differences in the T lymphocyte subsets involved. The purpose of this study was to perform a detailed examination of the immunomodulatory effects of topical CsA in conjunctival tissue from patients with AKC

Patients with active AKC were randomly allocated into two groups of four patients; one group received 2% CsA drops, and the other group received placebo drops, for 3 months. Superior tarsal conjunctival biopsies were harvested before and after treatment and examined by one- and two-colour IHC to compare leukocyte counts, HLA-DR$^+$ and IL-2R$^+$ cell counts, HLA-DR positivity of conjunctival epithelial cells and counts of T cell expressing IL-2, IL-3, IL-4, IL-5 and IFN-γ.

Post-treatment values were significantly less than pre-treatment values for the total number of leukocytes and in the numbers of CD3$^+$ T cells, CD4$^+$ cells, CD8$^+$ cells, CD20$^+$ B cells, neutrophils and macrophages, and there was a decrease in the CD4/CD8 ratio (p=0.03) in the cyclosporin A group. There was a reduction from pre-CsA-treatment to post-CsA-treatment in the numbers of HLA-DR$^+$ and IL-2R$^+$ cells (p=0.03) but the reduction in the epithelial cell HLA-DR expression did not reach significance. The numbers of T cell staining for IL-3 and IL-5 were reduced, although not to statistical significance, but there was a significant reduction in the number of T cells expressing IL-2 and IFN-γ (p=0.03) after CsA treatment compared to initial values. There were no statistically significant differences between pre-treatment and post-treatment values in the placebo group.
There was a clinical improvement in the CsA group and a clinical worsening in the placebo group.

The *in vivo* effects of CsA were shown to translate into a reduction in T cell numbers, a normalisation of CD4/CD8 ratio, a decrease in T cell activation and a reduction in T cell cytokine expression, especially IL-2 and IFN-γ. The decrease in HLA-DR expression may be explained by the reduction in IFN-γ. There were fewer B cells but not plasma cells after CsA and no change in IL-4 expression, suggesting minimal effects upon type I hypersensitivity responses. There was no significant reduction in mast cell or eosinophil numbers, but direct effects of topical CsA upon their function may play a role in the therapy of ocular allergic disease. These results show that the beneficial effects of topical CsA in AKC are accompanied by important changes in conjunctival immune cell profiles.

Conclusions

The pathogenesis of chronic allergic eye disease is very complex, with multiple cell types generating pro-allergic cytokines and each cell type showing functional differences between the different disorders. The mechanics of the intercellular interactions are not yet known and the relationship between clinical characteristics and variations in cellular function is uncertain. However, these variations may provide a basis for better prognostics and prevention and the development of future therapies which are safer than steroids. Potentially, it may be possible to construct therapies specifically tailored to the disease or even the individual patient based on cellular variations.

Research in the immediate future will use ISH to confirm the production of cytokines by eosinophils and epithelial cells, and culture of individual conjunctival cell types for examination of phenotype and cytokine production and intercellular interactions. Cellular variations can be compared with long-term disease outcomes to aid prognostication. Our research could be replicated in systemic allergic disorders for comparison and, if further similarities are found, the conjunctiva may prove a useful site as a model for asthma and AD inflammation. The results suggest certain therapeutic agents (e.g. anti-cytokines, anti-T cell preparations) could be assessed for topical ocular use in serious ocular allergy and may stimulate the development of new agents (e.g. anti-eosinophil drugs).

It is believed that the results presented here, by furthering our understanding of the pathogenesis of serious ocular allergic diseases, will provide the groundwork for the development of better therapy and prevention in the future.

Chapter 1

Introduction

1.1 Perspective

Ocular allergy, manifesting as inflammation of the conjunctiva (conjunctivitis) and sometimes inflammation of the lid skin and lid margins and disease of the cornea (keratopathy), is one of the most frequent diseases of the eye (Dart et al., 1986). The term encompasses a number of distinct disease entities with a broad spectrum of severity, ranging from mild, non-sight threatening disorders (e.g. seasonal allergic conjunctivitis) to more serious conditions (e.g. atopic keratoconjunctivitis, vernal keratoconjunctivitis) which, because of corneal involvement, are potentially blinding. To understand such disorders and investigate their immunopathogenesis, it is first necessary to understand the normal structure and function of the ocular surface, that is, of the eyelids, the cornea and particularly the conjunctiva, and also the nature of allergy and allergic responses.

1.2 The normal ocular surface

1.2.1 Macroscopic anatomy

1.2.1.1 The eyelids

The lids extend from the orbital rim to the palpebral aperture (Fig. 1.1). The anterior lamella consists of skin overlying orbicularis oculi muscle (responsible for lid closure) and the posterior lamella of the tarsal plate (dense connective tissue containing the meibomian glands) tightly adherent to conjunctiva (Fig. 1.2). The orbital septum is a fibrous sheet running from the orbital rim to the tarsal plate which separates the lid from the orbit. Levator palpebrae superioris (the main upper lid retractor) originates at the orbital apex and its aponeurosis passes through the orbital septum and inserts into the lid skin and the tarsal plate. Muller's muscle, another upper lid retractor, arises from the inferior surface of levator palpebrae and inserts in the upper border of the tarsal plate. In the lower lid, a sheet of fibrous tissue analagous to the levator aponeurosis arises from the sheath of the inferior ocular muscles, and the inferior tarsal muscle is analagous to Muller's muscle.

The mucocutaneous junction (grey line) runs horizontally along the lid margin dividing it into an anterior part containing the lash follicles, the glands of Zeis (modified sebaceous glands associated with the lash follicles) and the ducts of the glands of Moll (modified sweat glands) and a posterior part containing the meibomian gland orifices (Fig. 1.3). Approximately 25 of these modified sebaceous glands lie vertically in the tarsal plate and produce the superficial lipid layer of the precorneal tear film.

The lids have a rich network of arteries. The lateral and medial palpebral arteries (derived from the internal carotid) supply the marginal and peripheral vascular arcades.

There are profuse anastomoses with branches of the external carotid artery. Venous return is conveyed by the ophthalmic, angular and superficial temporal veins. Lymph drains to the submandibular and the preauricular nodes.

Lid sensation is mediated by branches of the ophthalmic division of the trigeminal nerve in the upper lids, and in the lower lids by branches of the maxillary division of the trigeminal nerve. The orbicularis oculi muscle is supplied by the facial nerve and the levator muscle by the oculomotor nerve; Muller's muscle and the inferior tarsal muscle are supplied by sympathetic nerves arising from the superior cervical ganglion.

1.2.1.2 The conjunctiva

The conjunctiva is a thin, semi-transparent, vascular mucous membrane lining the inner lid surfaces and the anterior sclera (Fig. 1.4). It is involved in host defences against infection and trauma and helps maintain a suitable environment for the cornea. The conjunctival epithelium is continuous with the corneal epithelium at the limbus and with the epidermis of the eyelid margin skin at the grey line. Clinically, the conjunctiva is divided into: the bulbar portion (from the limbus over the anterior sclera, loosely attached to underlying tissues), the forniceal portion (where the conjunctiva in reflected back towards the lids and lies in loose folds) and the tarsal conjunctiva (which lies on the inner surface of the lids and is firmly attached to the tarsal plate) (Fig. 1.5). The marginal conjunctiva is a 2mm wide strip that runs from the grey line of the lid margin to the subtarsal sulcus, a small groove running parallel and near to the lid margin and which acts as a trap for small particles to prevent corneal irritation.

The arterial supply of the tarsal and forniceal conjunctiva arises from the two palpebral arcades, and that of the bulbar conjunctiva from the anterior ciliary artery via a superficial and a deep plexus. The veins run with the arteries and drain into the palpebral veins or the superior and inferior ophthalmic veins. Lymph drainage parallels that of the lids. Sensation is mediated by branches of the ophthalmic (superior conjunctiva) and maxillary (inferior conjunctiva) divisions of the trigeminal nerve. Sympathetic fibres arise from the superior cervical ganglion and vasomotor parasympathetic fibres from the pterygopalatine ganglion.

1.2.1.3 The cornea

This is the transparent anterior part of the outer coat of the eye, which allows light to enter and which functions as the main refractive surface of the eye as well as being important in maintaining the anatomical integrity of the globe. In a normal emmetropic eye, the cornea is circular posteriorly with a diameter of 11.6 mm but anteriorly, the

superior and inferior encroachment of the limbal tissue means that the cornea appears elliptical with a vertical diameter of 10.6 mm. The corneal surface is aspherical, with a smaller radius of curvature centrally than peripherally.

The cornea is avascular and although peripherally the limbal circulation is important, the majority of metabolic needs are met by diffusion from the atmosphere/tear film and from the aqueous. Branches of the long and short ciliary nerves (ophthalmic nerve) anastomose with the conjunctival nerves to form a pericorneal plexus; from this a superficial and a deep group of nerves run into the cornea. The nerves become demyelinated 1-2 mm into the cornea and some fibres of the superficial group enter the epithelium and course between the cells.

1.2.1.4 The limbus

This is the transition zone between the cornea and the sclera plus conjunctiva. The limbus is 1-2mm wide and is broadest superiorly and inferiorly. It runs from the peripheral terminations of the Bowman's and Descemet's membranes (see below) to the scleral spur and contains the trabecular meshwork and canal of Schlemm, via which aqueous humour escapes the eye. At its posterior edge, numerous white interdigitations can be seen, known as the palisades of Vogt, which are crests of subepithelial vascular papillae separated by thickened epithelial downgrowths. The anterior conjunctival branches of the anterior ciliary artery run circumferentially in the limbus, giving off radial branches to the deep and superficial pericorneal plexuses.

1.2.2 Histology

1.2.2.1 The conjunctiva

The normal conjunctiva consists of a non-keratinising, stratified squamous epithelium between 2 and 10 cell layers thick, overlying a basement membrane and a substantia propria which consists of loose, highly vascular connective tissue. Leukocytes are concentrated in the anterior portion of the substantia propria, the so-called adenoid or lymphoid layer. The posterior portion of the substantia propria is more fibrous and is continuous with the tarsal plate, Tenon's membrane and the extraocular muscle tendons. Melanocytes are scattered in the conjunctiva and donate melanosomes to the conjunctival epithelial cells. The accessory glands of Wolfring and Krause in the substantia propria supplement the aqueous tear layer, the majority of which is produced by the lacrimal gland. Goblet cells in the epithelium secrete mucus which adheres to microvilli and microplicae on the surface of the epithelial cells; this helps to stabilise the tear film by converting a hydrophobic surface (the epithelial cell membrane) into a hydrophilic surface for the aqueous tear component. The tear film

consists of three layers: the mucin layer (0.5μm thick, from the goblet cells), adjacent to the ocular surface; the aqueous layer (85μm thick, from the lacrimal gland and the accessory lacrimal glands); the lipid layer (0.5μm thick, from the meibomian glands, which reduces the rate of tear evaporation). The tear film is redistributed evenly over the ocular surface with each blink of the eyelids. Besides maintenance of the normal moistness of the ocular surface, the functions of the tear film include providing optical uniformity for the corneal surface, flushing away cellular debris and foreign matter, nutrition for the ocular surface, and ocular defence against infection via the antibacterial effects of tears (mechanical, chemical e.g. lactoferrin, lysozyme and immunological e.g. complement, immunoglobulins).

1.2.2.2 The cornea

There are five corneal layers: epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium (Fig. 1.6). The epithelium has approximately 5 cell layers: a single layer of columnar cells lying upon the basement membrane, two layers of wing cells and, most superficially, flat cells which are shed into the tears and replaced from below. Bowman's layer is the dense, acellular, anterior portion of the corneal stroma. The stroma proper is made up of regularly-spaced parallel lamellae of collagen fibrils of uniform diameter surrounded by a glycosaminoglycan extracellular matrix and scattered cells (keratocytes). The regular arrangement of layers of collagen fibrils, running parallel to the corneal surface and at right-angles to each other, is required for the transparency of the tissue. Descemet's membrane is the basement membrane of the endothelium. The endothelium is a single cell layer which maintains the relative dehydration, and thereby the transparency, of the stroma by an active pump mechanism. These cells cannot replicate and cell loss can only be countered by enlarging of neighbouring cells.

1.2.2.3 The limbus

There is a gradual transition from the five-layered corneal epithelium to the ten-layered conjunctival epithelium, and the epithelium projects downwards between the subepithelial upward extensions. The basement membrane is continuous with that of the conjunctiva. Subepithelial tissue appears, which is continuous with the conjunctival substantia propria, and forms multiple upward projections, containing capillary loops and lymphatics, which are the palisades of Vogt. The regularity of the corneal lamellae gives way to the irregular diameter and arrangement of scleral fibres and the endothelium of the cornea continues as the endothelium of the trabecular meshwork.

1.2.3 Ocular surface defence and conjunctival leukocytes

1.2.3.1 Non-antigen-specific defence

Multiple non-specific mechanisms are involved in the defence of mucosal surfaces against pathogens. The cover of the bony orbit, lids and cilia (eyelashes), the flushing action and chemical composition of the aqueous tears (e.g. lysozyme, lactoferrin, prostaglandins, leukotrienes, interferons), the tear film mucus, spillover of the normal lid margin flora and the presence of natural killer (NK) cells, neutrophils and macrophages provide non-specific protection for the ocular surface (Chandler and Gillette, 1983; Smolin, 1985).

1.2.3.2 Antigen-specific defence of mucosal surfaces

As with other body surfaces in contact with the external environment, such as the skin or the mucous membranes of the gut and lung, the conjunctiva is constantly bombarded by a vast number of antigens and allergens (Chandler, 1996). Antigen-specific defence of mucosal surfaces is afforded by the constitutive presence of lymphoid cells, which quantitatively exceed those of the peripheral lymphoid organs (Roitt et al., 1993; Cerf-Bensussan and Guy-Grand, 1991; Beagley and Elson, 1992). Mast cells are also present in the mucosae in large numbers and have the ability to release many preformed and newly-synthesised mediators, with multiple effects on blood vessels and other leukocytes (Roitt et al., 1993; Watt and Ward, 1985).

1.2.3.3 Leukocytes in the normal conjunctiva

Even in the normal, non-inflamed conjunctiva, leukocytes are a frequent finding. The mean number of leukocytes in the epithelium is 20,000 cells/mm^3 (range 0-50,000 cells/mm^3) and in the substantia propria 154,000 cells/mm^3 (range 10,000-355,000/mm^3) (Allansmith et al., 1978). Allansmith (Allansmith et al., 1978) found greater numbers of leukocytes (including lymphocytes) in the tarsal conjunctiva compared with the lower fornix whereas Sacks (Sacks et al., 1986a) found the greatest number of lymphocytes in the fornix and least in the bulbar area. The pattern of cell type in the epithelium differs from that in the substantia propria; plasma cells and mast cells are not seen in the normal epithelium despite their presence in the normal substantia propria (Allansmith et al., 1978; Bernauer et al., 1993a; Irani et al., 1990). Mast cells lie mainly in relation to blood vessels and meibomian glands and 95% are of the MCtc (chymase and tryptase-containing) or connective tissue type (Irani et al., 1990; Morgan et al., 1991a). Neither the eosinophil nor the basophil constitute a normal conjunctival component (Allansmith et al., 1979; Allansmith et al., 1978).

T cells are the most frequent cell type, outnumbering B cells (Chan et al., 1988; Sacks et al., 1986a; Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982). Previously

published normal conjunctival CD4:CD8 ratios vary, but most commonly report a slight preponderance of $CD8^+$ cells (Chan et al., 1988; Sacks et al., 1986a; Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982). Most authors have found that the majority of epithelial T cells are $CD8^+$ (Chan et al., 1988; Sacks et al., 1986a; Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982; Dua et al., 1995; Soukiasian et al., 1992) and that the CD4:CD8 ratio is somewhat higher in the substantia propria. $CD4^+$ cells may outnumber $CD8^+$ (Chan et al., 1988; Foster et al., 1991; Dua et al., 1995) or the CD4:CD8 ratio may approximate to 1 with slightly more $CD8^+$ cells (Sacks et al., 1986a; Bernauer et al., 1993a; Bhan et al., 1982; Soukiasian et al., 1992). It may be that there is a crucial balance of $CD4^+$ and $CD8^+$ cells in the normal state, representing a physiological immunoequilibrium (Sacks et al., 1986a; Jakobiec et al., 1984); inflammatory conjunctival disorders are often associated with increased CD4:CD8 ratios in the epithelium and substantia propria (Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982; Dua et al., 1995; Soukiasian et al., 1992; Jakobiec et al., 1984; Power et al., 1993).

The number of plasma cells found in the normal conjunctiva is controversial (Bhan et al., 1982; Sacks et al., 1986a). Immunoglobulins are present in normal human tears, IgA being predominant (IgA>IgG>IgE>IgM), with more IgA plasma cells present than other plasma cell types (Little et al., 1969; Sompolinsky et al., 1982; McClellan et al., 1973). At least part of the IgA and IgE is produced locally (from plasma cells in the conjunctiva and the lacrimal gland) and this proportion increases in allergic disease (Aalders-Deenstra et al., 1985; Donshik and Ballow, 1983). As in other mucosal secretions, tear IgA is dimeric with a secretory component added by conjunctival and lacrimal gland epithelial cells (Sompolinsky et al., 1982).

1.2.3.4 Mucosal-associated lymphoid tissue

The term MALT (mucosal-associated lymphoid tissue) describes a specific arrangement of lymphoid tissue within mucosae. MALT is believed to occur in a number of mucosal sites of which the gut MALT is the best characterised (Cerf-Bensussan and Guy-Grand, 1991; Beagley and Elson, 1992; Tomasi et al., 1980). There are 3 main components to MALT (Roitt et al., 1993; Beagley and Elson, 1992). These are: 1) aggregates in the substantia propria (e.g. Peyer's patches) which may contain germinal centres and which lie directly under specialised flattened epithelium containing M (microfold) cells and a paucity of glandular elements; 2) scattered lamina propria lymphocytes (LPLs), mainly $CD4^+$ memory cells and some plasma cells, B cells and macrophages; and 3) intraepithelial lymphocytes (IELs) which are mainly $CD8^+$ memory T cells expressing HML-1 (human mucosal lymphocyte-1) antigen and

which lie between basal epithelial cells (Roitt et al., 1993; Cerf-Bensussan and Guy-Grand, 1991; Beagley and Elson, 1992). These lymphocytes are distinguished by their ability to produce (or promote the production of) immunoglobulin A (IgA), by their tendency to recirculate in the mucosal system, and by the phenomenon of ecotaxis (homing) (Roitt et al., 1993; Beagley and Elson, 1992; Tomasi et al., 1980; Bienenstock and Befus, 1984).

1.2.3.5 Conjunctival-associated lymphoid tissue

Animal studies have shown chickens, rabbits and guinea pigs to have a well-developed CALT (conjunctival-associated lymphoid tissue) with substantia propria lymphoid aggregates underlying specialised epithelium containing M cells (Franklin and Remus, 1984; Fix and Arp, 1991; Axelrod and Chandler, 1978; Latkovic, 1989). The human conjunctival substantia propria and epithelium do contain lymphocytes which have the properties of homing, mucosal recirculation and IgA production (Smolin, 1985; Chandler, 1996; Bloomfield, 1985). Conjunctival IELs share some of the properties of gut IELs: they are predominantly $CD8^+$, are found in the basal epithelial layers and bear HML-1. However, like other non-gastrointestinal mucosal sites, there is no increase in T cell receptor (TCR)γδ cell numbers in the conjunctiva (Roitt et al., 1993; Cerf-Bensussan and Guy-Grand, 1991; Beagley and Elson, 1992; Bienenstock and Befus, 1984; Chan et al., 1988; Sacks et al., 1986a; Bhan et al., 1982; Jakobiec et al., 1984; Dua et al., 1994 & 1995; Cerf-Bensussan and Guy-Grand, 1991; Soukiasian et al., 1992; Groh et al., 1989).

Stromal lymphocytes are clustered in the "adenoid layer" in the superficial part of the conjunctival substantia propria (Records, 1988; Bron et al., 1985). The substantia propria lymphocytes again show similar characteristics to substantia propria lymphocytes in other MALT, with more equal numbers of $CD4^+$ and $CD8^+$ cells than in the epithelium and frequent expression of CD45Ro (Beagley and Elson, 1992; Chan et al., 1988; Sacks et al., 1986a; Bhan et al., 1982; Schieferdecker et al., 1992). CALT aggregates with overlying specialised epithelium in close association have been demonstrated in humans (Dua et al., 1994; Wotherspoon et al., 1994) but other work suggests human CALT is not as well developed as that in animals (Wotherspoon et al., 1994).

1.2.3.6 Conjunctival antigen presenting cells

Conjunctival antigen presenting cells (APC), bearing MHC (major histocompatibility) class II molecules, are important for the transportation of antigen to regional lymph nodes and for their interaction with local lymphocytes, and include macrophages and

specialised APC (Smolin, 1985; Roitt et al., 1993; Sacks et al., 1986b; Foster et al., 1991). Langerhans cells are present in the conjunctival epithelium from birth (Chandler and Gilette, 1983; Chan et al., 1988; Sacks et al., 1986a; Bodaghi et al., 1994) and non-Langerhans dendritic cells (probably interdigitating dendritic cells) have been identified in the substantia propria (Chan et al., 1988; Sacks et al., 1986a & 1986b). It has been suggested that epithelial cells in other mucosae may present antigen under certain circumstances (Devalia and Davies, 1993).

1.3 Allergy and atopy

1.3.1 The concept of allergy

Allergy is an exaggerated or abnormal response of the immune system to external, non-infectious substances (known as allergens) which come into contact with a body surface (skin or mucosal surface) or are injected. Allergic diseases recognised by the World Health Organisation are rhinitis, sinusitis, asthma, hypersensitivity pneumonitis, extrinsic allergic alveolitis, conjunctivitis, urticaria, eczema and atopic dermatitis, contact dermatitis, anaphylaxis, angiooedema, allergic and migraine headache and certain gastrointestinal disorders (food allergy) (Thompson and Stewart, 1993).

1.3.2 Hypersensitivity reactions

Hypersensitivity reactions describe any exaggerated or abnormal response of the immune system to either external (infectious or non-infectious) or internal (self-) antigens, and have been classified into four types (Coombs and Gell, 1963). In type I (immediate) hypersensitivity, the initial contact with allergen results in IgE production by plasma cells, which binds to the specific, high affinity $Fc_{\varepsilon}R1$ receptor of mast cells present in mucosal tissues and skin; at this point no clinical reaction occurs. On re-exposure to the same allergen, binding of multivalent allergen causes cross-linking of mast cell surface IgE, which triggers mast cell degranulation, releasing both preformed, granule-stored mediators (histamine, tryptase, chymase, carboxypeptidase A, heparin, eosinophil- and neutrophil chemotactic factors etc.) and newly-synthesised lipid mediators (leukotrienes [LTs], prostaglandins [PGs], platelet activating factor [PAF], thromboxanes [Tx] hydroxyeicosatetraenoic acids [HETEs] etc.) (Serafin and Austen, 1987; Holgate, 1991; Goldstein, 1994). These mediators have potent proinflammatory effects, including vasodilatation, increased vascular permeability, increased mucus production, induction of adhesion molecule expression, and leukocyte chemotaxis and activation (Serafin and Austen, 1987; Holgate, 1991; Goldstein, 1994; Holgate, 1988). Examples of type I hypersensitivity are the reaction to diagnostic skin prick tests and anaphylactic shock.

Type II (cytotoxic) hypersensitivity reactions are initiated by antibody reacting with a cell surface antigen. This usually involves complement activation and an example is incompatible blood transfusion reaction. In a subtype, type IIb (cell-stimulating) reaction, an antibody against a cell surface receptor has a cell stimulatory (agonist) effect (e.g. Grave's disease, where IgG antibody against thyroid-stimulating hormone receptor simulates thyroid hormone production). Type III (immune complex) hypersensitivity reactions occur when antigen and antibody form complement-activating complexes in and around the microvasculature, which are toxic to cells. Examples include immune-complex glomerulonephritis and serum sickness.

Type IV (delayed-type) hypersensitivity reactions occur when sensitised T lymphocytes react with antigen at a tissue site to produce cell damage with a macrophage-rich infiltrate. The classical (type IVa) delayed-type reaction involves predominantly $CD4^+$ T cells and MHC class II-restricted antigen presentation, and examples are the tuberculin reaction, contact dermatitis and rheumatoid arthritis (Janeway and Travers, 1995). Type IVb responses involve cytotoxic $CD8^+$ T cells and an MHC class I-restricted antigen presentation stimulating apoptosis of cells; examples of this are early onset, insulin-dependent diabetes mellitus and graft rejection (Janeway and Travers, 1995).

1.3.3 Atopy

The term atopy was first used to describe a tendency to develop type I hypersensitivity reactions to common allergens (Coca and Cooke, 1923). Atopic individuals have a genetic predisposition for inappropriate synthesis of IgE antibody specific for normally harmless environmental allergens (e.g. pollen, house dust mite) and develop immediate hypersensitivity responses after exposure to allergen (e.g. a positive wheal-and-flare reaction after skin-prick tests). However, some patients with atopic diseases (asthma, rhinitis, atopic dermatitis [AD]) may have negative skin-prick tests and normal serum levels of IgE antibody, whilst in others the presence of allergen-specific IgE is not associated with disease. Atopy has been linked to an abnormal locus at chromosome 11q (Cookson et al., 1989; Sandford et al., 1993; Hill et al., 1995) and also to loci on chromosome 5 close to the genes that encode interleukin (IL)-4 and control IgE levels (Marsh et al., 1994; Meyers et al., 1994). There are also associations between human leukocyte antigen (HLA)-class II phenotype and IgE response to common allergens (Young et al., 1994).

1.3.4 The immunopathogenesis of allergic disease

Allergy and allergic diseases are traditionally described as type I (immediate) hypersensitivity responses. However, in modern histopathological studies, examination of biopsies and lavage fluids in asthma, allergic rhinitis and atopic dermatitis has shown chronic mucosal or skin inflammation. There is epithelial destruction, expansion and activation of fibroblasts, and hypertrophy of smooth muscle, with leukocyte infiltration into tissue and increased numbers of leukocytes in lavage fluids. Elevated numbers of eosinophils, and increased numbers of activated and memory $CD4^+$ T cells predominate (other cells present include degranulated mast cells and macrophages) and appear to correlate with epithelial damage and clinical measures (Wardlaw et al., 1988; Azzawi et al., 1990; Bradley et al., 1991; Hamid et al., 1992a; Robinson et al., 1993a; Laitinen et al., 1985; Bentley et al., 1992; Varney et al., 1992; Leung, 1992; Lever et al., 1987; Leiferman, 1994).

When a sensitised individual undergoes challenge with allergen, the clinical response (e.g. bronchoconstriction after inhalational challenge, wheal-and-flare response after skin prick challenge) consists of an early phase response (EPR, early asthmatic response, early cutaneous response etc.) at 15-20 minutes lasting up to 1 hour and, frequently, a second, delayed, late-phase response (LPR) at 4 to 6 hours (Herxheimer, 1952). The EPR appears to be due to a classic type I hypersensitivity response mediated by products of mast cell degranulation (both preformed and newly-synthesised mediators) (Serafin and Austen, 1987; Holgate, 1991; Goldstein, 1994). The LPR has been used as a model of chronic allergic inflammation. Biopsy of tissue (e.g. bronchial, skin biopsies) and investigation of cells in lavage fluids during the LPR also show inflammatory changes, with an eosinophil and $CD4^+$ T cell-rich cellular response (Frew and Kay, 1988; Gaga et al., 1991; Durham et al., 1992). Thus it appears that allergic disease and the response to allergen involve a form of type IVa hypersensitivity and this has been subclassified as type IVa_2 hypersensitivity (chronic allergic inflammatory tissue reaction or cell-mediated eosinophilic hypersensitivity) (Kay, 1997).

1.3.5 The role of cytokines in allergy

1.3.5.1 Cytokines

Cytokines are soluble proteins or glycoproteins produced by leukocytes (and other cell types) which allow local chemical communication between cells in homeostasis and pathology. They often show both pleiotropy and redundancy. Until recently, T cells were believed to be the major source of cytokine in allergic inflammation (see below).

1.3.5.2 Control of IgE synthesis: IL-4 and IL-13

Cytokines play an important role in regulating the amount and the type (isotype switching) of antibody production. In humans, IL-4 promotes the growth and differentiation of B cells and induces B cells to switch to the synthesis of IgE and IgG4 (DelPrete et al., 1988; Pene et al., 1988a; Gascan et al., 1992); it also increases B cell expression of certain surface antigens, including CD23, CD40 and HLA class II molecules (Fisher et al., 1990; Rousset et al., 1988). IL-13 can also cause switching to IgE synthesis in human B cells (Punnonen et al., 1993a). This process requires additional signals including cell-cell contact between activated $CD4^+$ cells and B cells (via CD40/CD40 ligand interaction and CD21/CD23 interaction) (Jabbara et al., 1990; Aubry et al., 1992). Other T cell-derived cytokines which facilitate B cell activation and clonal expansion, and promote the IL-4-induced IgE synthesis, are IL-2, IL-5, IL-6 and tumour necrosis factor (TNF)-α (Pene et al., 1988b; Vercelli et al., 1989; Maggi et al., 1989; Gauchat et al., 1992). However, other cytokines are inhibitory to IgE synthesis. Interferon (IFN)-γ reduces IL-4-induced IgE production in humans (DelPrete et al., 1988; Pene et al., 1988a) and other cytokines that have an inhibitory effect include IL-8 (Kimata et al., 1992), IL-12 (Kinawa et al., 1992) and IL-10 (Punnonen et al., 1993b).

1.3.5.3 Cytokines in eosinophil recruitment and activation: IL-3, IL-5 and GM-CSF

There is a great deal of evidence to suggest an important role for eosinophils in allergic disease (see above and below) and there seems to be a specific accumulation of eosinophils in tissue. Eosinophil differentiation is influenced by T cell-derived cytokines, and IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) promote eosinophil maturation, activation and prolonged survival (Rothenberg et al., 1988a and 1989; Lopez et al., 1986). IL-5 acts specifically on eosinophils (Lopez et al., 1988) and transgenic mice overexpressing IL-5 show a marked blood and tissue eosinophilia (Dent et al., 1990). There are a number of non-specific eosinophil chemoattractants (e.g. PAF, IL-8, lymphocyte chemoattractant factor [LCF]) and IL-5, IL-3 and GM-CSF (themselves weakly chemoattractive for eosinophils) prime eosinophils for an enhanced response to such compounds (Wardlaw et al., 1986a; Rand et al., 1991; Warringa et al., 1991; Sehmi et al., 1992). Other important chemoattractants for, and activators of, eosinophils include the cytokines RANTES (Regulated upon Activation, Normal T cells Expressed and Secreted), monocyte chemotactic protein (MCP)-4 and MCP-3, macrophage inhibitory protein (MIP)-1α and eotaxin (Baggiolini and Dahinden, 1994; Jose et al., 1994).

Leukocyte migration into tissue involves interactions between receptors on their cell surface with ligands on the surface of vascular endothelial cells (adhesion molecule interactions). For eosinophils, important adhesion molecule interactions include the selectins (initial weak adhesion), intercellular adhesion molecule (ICAM)-1/leukocyte function antigen (LFA)-1 (firm adhesion) and vascular cell adhesion molecule (VCAM)-1/very late antigen (VLA)-4 (firm adhesion) (Wardlaw et al., 1994; Weller, 1992). IL-3 and IL-5 appear to specifically upregulate eosinophil adhesion to unstimulated endothelial cells via ICAM-1 (Walsh et al., 1990; Wardlaw et al., 1994). VLA-4 is expressed specifically by eosinophils (not neutrophils) and the VLA-4 /VCAM-1 interaction may underlie the specific eosinophil recruitment in allergic diseases (Walsh et al., 1991; Wardlaw et al., 1994); it may be important also in the adhesion of eosinophils to tissue fibronectin, which prolongs their survival (possibly by autocrine cytokine production) (Elices et al., 1990; Anwar et al., 1993). The expression of VCAM-1 on endothelial cells is increased by IL-4, which specifically increases the adhesion of eosinophils to endothelial cells (Schleimer et al., 1992).

1.3.5.4 T cell cytokines and mast cells

Mast cells circulate in the blood only as progenitors and local stem cell factor (SCF) is required for maturation of mast cells in the tissues (Galli et al., 1993; Nilsson et al., 1993). Growth and differentiation of mast cells are influenced by IL-3 and IL-4. IL-3 stimulates bone marrow precursors to differentiate into a mucosal mast cell phenotype in the mouse and this is potentiated by IL-4 (Tsuji et al., 1988). IL-10 also enhances mast cell growth, in synergy with IL-3 and IL-4 (Thompson-Snipes et al., 1991), and IL-9 increases the proliferative response of murine mast cells to IL-3 (Hueltner et al., 1990). However, IFN-γ appears to inhibit murine mast cell growth and activation (O'Hehir et al., 1991).

1.3.5.5 The identification of cytokines in allergic disease

Evidence that cytokines play an important role in allergic disease has also come from the detection of cytokine protein and mRNA in tissue or inflammatory fluid from patients with active disease. IL-5 mRNA has been shown in bronchial biopsies from asthmatics but not controls and the amount correlated with the number of eosinophils (Hamid et al., 1991). Robinson (Robinson et al., 1992) showed an increase in the percentage of bronchoalveolar (BAL) cells from asthmatics expressing IL-2, IL-3, IL-4, IL-5 and GM-CSF compared with normal subjects and the majority of the cells were T cells. Expression of these cytokines was proportional to the severity of the asthma (Robinson et al., 1993b). An increased percentage of blood T cells from patients with asthma expressed mRNA for IL-3, IL-4, IL-5 and GM-CSF compared with normal

subjects and this was reduced after treatment in line with clinical improvement (Corrigan et al., 1995). In rhinitis, expression of mRNA for IL-4 and IL-5 was detected and the predominant source was T cells (Durham et al., 1992; Ying et al., 1994, 1995). Treatment and clinical improvement were associated with a reduction in IL-4 mRNA expression (Masuyama et al., 1994). Cutaneous allergen challenge in atopic subjects is associated with abundant expression of mRNA for IL-4 and IL-5 (Kay et al., 1991). This all suggests that $CD4^+$ T cell expression of cytokines (e.g. IL-3, IL-4, IL-5, and GM-CSF) which promote eosinophil (and mast cell) activation and function occurs in allergic disease and that treatment may act at least in part by reducing the production of such cytokines.

1.4 T cells and allergy

1.4.1 T cells

T cells comprise the majority of peripheral blood lymphocytes and also reside in the lymphoid organs (e.g. spleen, thymus) and in mucosae. T cells express a number of cell surface antigens, including the TCR and other antigens, often described by cluster determination (CD) numbers. All mature, post-thymic T cells express CD2 and CD3. CD2 binds to the ligand LFA-3, an adhesion molecule. CD3 consists of four polypeptide chains closely associated with the TCR and is involved in transmitting the activation signal arising from antigen binding into the cell interior.

1.4.2 Phenotypically distinct T cell subsets

1.4.2.1 The T cell receptor

In the majority of T cells, the TCR is composed of two covalently-linked polypeptide chains, α and β. In a minority (1-10% peripheral blood T cells) the TCR is composed of different γ and δ chains (Brenner et al., 1986). T cells are not stimulated by TCR binding to antigen alone and recognise antigen only in association with MHC molecules.

1.4.2.2 CD4 and CD8 T cells

TCR$\alpha\beta$ T cells may be subdivided by their expression of CD4 and CD8 molecules. $CD4^+$ cells have been called T helper (Th) cells because they interact with B cells to promote antibody production. However, this term is somewhat misleading, as $CD4^+$ cells can also function as suppressor and cytotoxic cells (Morimoto et al., 1985; Patel et al., 1988). $CD4^+$ T cells recognise antigen in association with MHC class II antigens, found principally on the surface of antigen-presenting cells (e.g. macrophages, dendritic cells, B cells). Other cells (e.g. endothelial cells, epithelial

cells) can also be induced to express MHC class II antigen under certain circumstances, although their role in antigen presentation is unclear.

$CD8^+$ T cells recognise antigen in association with MHC class I antigen, which is expressed on virtually all nucleated cells. $CD8^+$ T cells are therefore able to recognise and kill self-cells expressing foreign antigen (malignant cells or virus-infected cells) but can also recognise cells bearing non-self histocompatibility antigens.

1.4.2.3 TCRγδ cells

TCRγδ T cells usually lack CD4 and CD8 expression, although a small proportion are $CD8^+$ (e.g. gut mucosa), and their cytotoxicity is not MHC class I restricted. Due to their propensity for mucosal surfaces, it is believed they may be involved in mucosal defence and inflammation (Janeway et al., 1988).

1.4.2.4 Activation markers

Expression of certain surface antigens generally occurs, or is increased, after activation of T cells by specific antigen, and implies cytokine secretion. Such antigens include the IL-2 receptor (IL-2R, CD25) and MHC class II antigens (both early expression) and VLA-1 and-2 (late expression)

1.4.2.5 Naive and memory T cells

The CD45R antigen (leukocyte common antigen, LCA) is found on both $CD4^+$ and $CD8^+$ cells and two of its structural forms, CD45RA and CD45RO, are formed by differential splicing of mRNA transcript. The degree of their expression on T cells is usually reciprocal and activation of T cells causes a reduction in expression of CD45RA and upregulation of CD45RO, a change which persists over time (Akbar et al., 1988). Such $CD45RO^+$ cells provide improved help for B cell immunoglobulin production, and show increased responses to antigen previously encountered, enhanced cytotoxicity, rapid production of a large range of cytokines and increased expression of other surface molecules (CD2, LFA-1, LFA-3, CD44) (Sanders et al., 1988a). CD45RO is therefore thought to be a marker for memory T cells, activated by previous exposure to antigen (Sanders et al., 1988b; Akbar et al., 1988; Akbar et al., 1991; Merkenschlager and Fisher, 1991).

However, there is some evidence that the relationship of CD45 isoform expression to memory type cell is more complex. It has been shown that $CD45RO^+$ and $CD45RA^+$ phenotypes may be reversible in humans (Rothstein et al., 1991) and that CD45RA expression is maintained on long-term cultured T cell lines and in the blood of adult

thymectomised patients (Sugita et al., 1992). Human T cell lines can also be CD45RO and CD45RA double positive in humans (Rothstein et al., 1991) and mice (Luqman et al., 1991). In mice, although not yet in humans, a correlation has been found between $CD45RO^+/RA^+$ T cells and Th2 subsets, and $CD45RO^+/RA^-$ with Th1 cells (see below, Luqman et al., 1991). However, the presence of CD45RO is still considered a reasonable means of identifying memory cells.

1.4.2.6 Adhesion molecules

T cells can express VLA molecules, LFA-1 (ligand for ICAM-1) and, if activated, also LFA-3 (ligand for CD2) and therefore T cells have the ability to bind to endothelial cells and to other leukocytes.

1.4.3 Functional T cell subsets

In recent years, it has become clear that distinct subsets of CD4 T cells can be identified on the basis of their cytokine production and that the nature of certain inflammatory disorders is, at least in part, dependent upon preferential activation of a particular subset of T cells.

1.4.3.1 Th1 vs. Th2 cells

Mosmann initially described two distinct subsets of $CD4^+$ T cell clones in long-term cloned murine T cell lines. Type I or Th1 cells secreted predominantly IL-2, IFN-γ and TNF-β and type II or Th2 cells secreted predominantly IL-4, IL-5, IL-6 and IL-10 (Mosmann et al., 1986; Mosmann and Moore, 1991). Other cytokines (TNF-α, GM-CSF) were produced by both Th cell types. A third population of cells, Th0, has also been identified, which produces a mixed pattern of cytokines (Firestein et al., 1989), and which may represent a third subset or a precursor early memory cell produced after initial antigen activation (Firestein et al., 1989; Swain et al., 1990).

In humans, it appeared that there was not such a clear-cut dichotomy in T cell subsets (Paliard et al., 1988; Andersson et al., 1988) but when human T cell clones were raised using specific antigens which stimulated classical delayed-type hypersensitivity (e.g. Mycobacterial antigen, nickel) or IgE-mediated responses (allergens, parasites), Th1-like (IL-2, IFN-γ, TNF-α) and Th2-like (IL-4, IL-5, IL-13) clones were inducible (Del Prete et al., 1991a; Yssel et al., 1991; Parronchi et al., 1991). IL-3, IL-6, IL-10 and GM-CSF were produced by both cell types although there was greater production by Th-2-like cells (Del Prete et al., 1994). Similar functional subsets were also discovered in human $CD8^+$ clones (Salgame et al., 1991).

These differences in cytokine production result in different effector functions (Mosmann and Coffman, 1989). Th1 cells are involved in delayed type (IV) hypersensitivity (Cher and Mosmann, 1987). Through secretion of IL-2 and IFN-γ, they promote the development of cytotoxic T cells, enhance the bactericidal activity of macrophages and stimulate activity of natural killer and LAK cells (Nagarkatti et al., 1990; Issekutz et al., 1988). Th1 cells can also provide help for immunoglobulin synthesis of isotypes IgM, IgG (especially IgG2a) and IgA but not IgE, although in conditions of T cell/B cell ratios greater than one, this is inhibited (DelPrete et al., 1991b), which may be a method of downregulation of the Th1 immune response. IFN-γ is known to inhibit IgE synthesis (DelPrete et al., 1988; Pene et al., 1988a). These functions of Th1 cells and their cytolytic action would be expected to be important for the elimination of viruses and other intracellular pathogens (Del Prete et al., 1994).

Th2 cells provide excellent help for B cells, enhancing the synthesis of immunoglobulins including IgE at all T cell/B cell ratios (Del Prete et al., 1991b) and are not cytolytic (Romagnani, 1995). This, together with their production of IL-5 along with IL-3 and GM-CSF, which stimulate eosinophils, and their preferential stimulation by allergens and parasites, would suggest their involvement in IgE-mediated and allergic disorders and in the defence against parasites and extracellular pathogens (Del Prete et al., 1994; Romagnani, 1995). It is possible that Th2 cytokines, by inhibition of cell-mediated immune responses, may be detrimental to host defence against certain pathogens. IL-4 inhibits the IL-2-dependent proliferation of Th-1 cells, and inhibits the effect of IFN-γ in enhancing the antimicrobial activity of monocytes (Lehn et al., 1989). IL-10 inhibits antigen presentation to T cells by monocytes and macrophages by down-regulating their MHC class II expression (De Waal-Malefyt et al., 1991a; Fiorentino et al., 1991) and also inhibits macrophage release of antimicrobial cytokines (De Waal-Malefyt et al., 1991b). That this might be relevant to human disease is suggested by the fact that in leprosy, tuberculoid leprosy (high immunity, restricting pathogen multiplication) Th1-type cytokine (IL-2 and IFN-γ) mRNA is prominent in skin lesions whereas as in lepromatous leprosy (low host immunity with uncontrolled proliferation of M. leprae) skin lesions have a predominance of Th2-type cytokine (IL-4, -5, IL-10) mRNA (Yamamura et al., 1992).

1.4.3.2 Cross-regulation of Th1 and Th2 cell subsets

There is evidence for the mutual cross-inhibition of proliferation and cytokine secretion by Th1 and Th2 lymphocytes, mediated by their cytokines. Exogenous IL-4 promotes the development into, and proliferation of, Th2 cells, and inhibits IL-2-dependent proliferation of Th1 cells and development of Th1 cells' cytolytic potential (Martinez et

al., 1990; Parronchi et al., 1992) but IFN-γ promotes differentiation into Th1 cells and inhibits proliferation of Th2 cells (Maggi et al., 1992; Gajewski and Fitch, 1988). In mice, IL-10 inhibits the proliferation and cytokine secretion of Th1 cells (Fiorentino et al., 1989; Fiorentino et al., 1991), although in humans IL-10 is produced by both Th1-like and Th2-like clones and inhibits proliferation of both T cell subsets (Del Prete et al., 1993a). In addition, Th1 and Th2 cells appear to inhibit each other's functions (see preceding paragraph).

1.4.3.3 Factors influencing differentiation into Th1 or Th2 cell types

It appears that Th1-like and Th2-like T cell subsets can occur in humans. In animals, their development can be influenced by the nature of the antigen, and certain antigens (e.g. Freund's adjuvant, bacterial antigens) result in Th1 cell clones whereas other antigens (e.g. alum adjuvant with Bordetella pertussis, helminths) induce a Th2 response (Mosmann and Coffman, 1989; Henderson et al., 1991). The dose of antigen can also have an important influence (DeKruyff et al., 1992). The nature of the antigen-presenting cell may also influence the differentiation of T cell subsets. B cells acting as APC tend to promote Th2 clones whereas macrophages acting as APC optimally stimulate Th1 clones (Gajewski et al., 1991; DeKruyff et al., 1992). The route of antigen-exposure may also affect the T cell subset development depending on where antigen is presented to T cells (e.g. mucosal, lymph node, spleen), which again may be partly related to APC type (Daynes et al., 1990; DeKruyff et al., 1992).

There are also genetic influences upon T cell subset differentiation. In mice, different strains can produce different responses to the same stimulus, such as Leishmania, where a Th2 response leads to death but a Th1 response leads to recovery, and MHC genotype also influences the type of Th response (Murray et al., 1992; Scott et al., 1989; Liew, 1989).

Similarly, in humans, the nature and identity of the stimulating antigen alone does not determine the subset type, as T cell clones with the same antigen-specificity can be Th1-, Th0- and Th2-type (Parronchi et al., 1991). Exogenous cytokines are able to influence the differentiation process in T cells. Exogenous IL-4 shifts the differentiation of peripheral blood lymphocytes stimulated with PPD from a Th1- to a Th0- or Th2- type, whereas addition of IFN-γ promotes differentiation into Th0 and Th1 clones (Maggi et al., 1992). Also, the cytokines IL-12 and IFN-α (products of macrophages which induce NK cells to secrete IFN-γ) also promote differentiation of PPD-stimulated T cells towards a Th0- or Th1-like phenotype, whereas anti-IL-12 antibodies promote a Th2-like phenotype (Romagnani, 1992). This has led to the

hypothesis that in viral and intracellular bacterial infection, pathogen presence in macrophages causes release of IL-12 and IFN-α which stimulate NK cells to release IFN-γ, promoting a Th1-like response (Romagnani, 1992). In parasite and atopic disease, IL-4 would be released from IgE-sensitised mast cells (see below) which would promote Th2 and inhibit Th1 development directly, and little IL-12 and IFN-α would be released from non-infected monocytes. Th2-like cells would produce more IL-4 and IL-13 (Zurawski and De Vries, 1994), which inhibit secretion of IFN-α and IL-12 from monocytes (De Waal-Malefyt et al., 1993) and thus there would be further differentiation into Th2-like cells and less Th1-like development. This cytokine balance would probably be affected by many host factors, including genetic, current immunological status and other influences. In reality, it is likely that human Th1 and Th2 cells coexist in a delicate balance which, when perturbed, results in disease (Kelso, 1995).

1.4.4 Evidence for Th2-like T cells in human allergic disease

Examination of tissue biopsies, lavage fluids and peripheral blood lymphocytes from subjects with allergic diseases such as asthma, AD and allergic rhinitis has demonstrated the presence of predominantly Th2-type cytokines.

In asthma, IL-5 mRNA was found in bronchial biopsies from asthmatics (correlating with numbers of infiltrating T cells and eosinophils) (Hamid et al., 1991) but not controls. In BAL cells in asthmatics, but not controls, there was elevated expression of mRNA for IL-2, IL-3, IL-4 and IL-5 but not IFN-γ, and the expression of these cytokines correlated with clinical severity (Robinson et al., 1992; Robinson et al., 1993b). There was greater peripheral blood $CD4^+$ lymphocyte expression of mRNA for, and secretion of, IL-3, IL-4, IL-5 and GM-CSF but not IFN-γ and IL-2 compared with controls (Corrigan et al., 1995) and this reduced with treatment in line with clinical improvement. Oral steroid treatment of asthmatics resulted in a reduction in BAL cell expression of IL-4 and IL-5 and an increase in expression of IFN-γ associated with clinical improvement (Robinson et al., 1993c) whereas allergen challenge in asthma was associated with increased expression of mRNA for IL-5 and GM-CSF in bronchial biopsies (Bentley et al., 1993). This contrasts with the Th1-like profile seen in pulmonary tuberculosis (Robinson et al., 1994).

In allergic rhinitis, Th2-type cytokines (IL-4 and IL-5) predominate at the mRNA level in nasal biopsies (Bentley et al., 1992; Durham et al., 1992; Varney et al., 1992; Ying et al., 1994 & 1995) and therapy is associated with a reduction in IL-4 mRNA expression (Masuyama et al., 1994).

In atopic allergen challenge to the skin, there is a predominance of mRNA for Th2-type cytokines (IL-4 and IL-5) but little IL-2 and IFN-γ, with the opposite profile in skin biopsies in the tuberculin reaction, a delayed type hypersensitivity response (Kay et al., 1991; Tsicopoulos et al., 1992).

More evidence for a role of Th2-like cells in allergic disease has come from the study of T cell clones derived from tissue lesions in such disorders. T cell clones derived from the skin in atopic dermatitis are mainly Th2-like in their cytokine profile (Van der Heijden et al., 1991; Ramb-Lindhauer et al., 1991; van Reijsen et al., 1992) as are T cell clones from mucosal biopsies of patients with allergic rhinitis or asthma (after allergen challenge) and these clones can stimulate IgE synthesis from autologous B cells (Del Prete et al., 1993b).

1.5 Mast cells and allergy

1.5.1 Mast cells

Mast cells are tissue cells which are widely distributed in the body, including the skin and mucosal surfaces, and are associated with nerves and blood vessels. Mast cells originate in the bone marrow (stimulated by IL-4), circulate in the blood as progenitors and only fully mature in the tissues, under the influence of tissue factors, particularly SCF.

1.5.2 Mast cell heterogeneity

Distinct subpopulations of mast cells were first identified in rodents, in which connective tissue mast cells are found in the skin and peritoneal cavity and mucosal mast cells are located in mucosae of the gut and lung (Enerback, 1966; Everitt and Neurath, 1980). The two types of mast cells were shown to have differences in their development, histochemical, biochemical, immunological and functional properties, as well as differences in localisation, suggesting an "immune role" for mucosal but not connective tissue mast cells (Table 1.1, reviewed by Rothenberg et al., 1988b; Holgate, 1991; Church et al., 1994).

Human mast cells are also heterogeneous in their staining properties (Strobel et al., 1981), but there appears to be a less absolute correlation between anatomical location and biochemical/functional properties (Befus et al., 1985; Marshall et al., 1987). Human mast cells have therefore been classified by their neutral protease content into MCt (tryptase-only containing mast cells) and MCtc (tryptase and chymase-containing mast cells, also contain chymase, carboxypeptidase and cathepsin G) (Irani et al., 1986,

1989a & 1991; Schecter et al., 1990). Certain parallels exist between the rodent and human mast cell divisions: MCt are preferentially located at mucosal sites (Irani et al., 1989b) and appear to be involved in immune functions, as they increase in number in allergic disease (Irani et al., 1989b & 1990) and reduce in number in immunodeficiency syndromes (Irani et al., 1987); MCtc are located preferentially at connective tissue sites and their numbers are not changed in allergic and immunodeficiency states (Irani et al., 1987, 1989a, 1989b & 1990). However, the situation in humans appears to be much more complex, with mast cells from different tissue sites demonstrating different functional properties (e.g. response to IgE-dependent degranulation and response to drugs such as cromoglycate) independent of their neutral protease subtype (Lowman et al., 1988a; Church et al., 1991).

1.5.3 Mast cell mediators

Mast cells can be stimulated to release their mediators in a variety of ways. Immunological stimulation, via cross-linkage of membrane IgE bound to high affinity IgE receptors (FcεR1) leads to activation of the phosphatidyl-inositol cycle and an increase in intracellular calcium (both from an influx of extracellular calcium and release of intracellular calcium stores) which results in compound exocytosis, leading to release of preformed mediators and synthesis of arachidonic acid-derived mediators (Rothenberg et al., 1988b; Holgate, 1991). Human mast cells from mucosal sites appear to be almost exclusively triggered in this way (Lowman et al., 1988a). However, mast cells derived from connective tissue sites (skin) can be stimulated by other stimuli, such as substance P, complement components C5a and C3a, opiates, compound 48/80 and eosinophil granule proteins (Lowman et al., 1988a). This mediator release differs from that stimulated by IgE cross-linkage, in that it does not require extracellular calcium, is more rapid and results in much less production of newly-synthesised mediators (Lowman et al., 1988b; Benyon et al., 1989).

The secretory granules of the mast cell contain histamine and proteases bound to proteoglycan. Of the preformed mast cell mediators, histamine is the most abundant and exerts many potent effects (via at least three histamine receptors) including vasodilatation, increased vasopermeability, itch, mucus production and contraction of bronchial smooth muscle (Serafin and Austen, 1987; Goldstein, 1994; Holgate, 1991 & 1988). The proteoglycan content of human mast cells is heparin (mainly) and chondroitin sulphates and its main function appears to be in binding preformed granule mediators and influencing their degree of inactivation (Holgate, 1988). Tryptase is present in all human mast cells and has a number of potentially important actions, including enzymatic effects upon neuropeptides, matrix component digestion and

sensitisation of bronchial smooth muscle (Tam and Caughey, 1990; Sekizawa et al., 1989; Walls et al., 1995). It also has kininogenase activity (Proud, Siekierski and Bailey, 1988), can be mitogenic for fibroblasts (Hartmann et al., 1992) and is chemotactic and secretogenic for eosinophils and mast cells (Walls et al., 1995). Chymase has enzymatic effects on neuropeptides, collagen, angiotensin I (conversion to angiotensin II) and cytokines (Urata et al., 1990; Sage et al., 1979; Mizutani et al., 1991).

Immunologically-stimulated mast cells also metabolise arachidonic acid via the cyclo-oxygenase and lipoxygenase pathways to produce PGD2 and LTC4, LTD4 and LTE4 (the latter two indirectly), which are powerful bronchoconstrictors, chemokinetic for neutrophils, increase vasopermeability and stimulate mucus production (Rothenberg et al., 1988b; Holgate, 1988; Holgate, 1991). PAF is also generated and has effects including platelet aggregation, vasodilatation, increased vascular permeability, granulocyte chemotaxis and eosinophil activation and degranulation (Kroegel, 1992; Wardlaw et al., 1986b).

1.5.4 Mast cell cytokines

In recent years it has been established that mast cells are a source of cytokines. Initial studies in rodents demonstrated that mast cell lines and cultures were able to synthesise and release IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, IFN-γ, TNF-α, MIP-1α and MIP-1β (Chung et al., 1986; Brown et al., 1987; Burd et al., 1989; Plaut et al., 1989; Gordon et al., 1990). In humans, a number of cytokines have been localised to mast cells. IL-4 immunoreactivity and mRNA has been localised to mast cells in nasal, lung and skin tissue (Bradding et al., 1993; Bradding et al., 1992; Ying et al., 1994). Following this, other cytokine protein and mRNA localised to human mast cells (either constitutively, after stimulation, or in disease) include IL-3, IL-5, IL-6, IL-8, IL-13, GM-CSF, TNF-α and others (Bradding et al., 1993; Bradding et al., 1994; Ohkawara et al., 1992; Okayama et al., 1995; McNamee et al., 1991) and patterns of mast cell cytokine suggest the possibility of mast cell heterogeneity for this characteristic too.

1.5.5 Mast cells in allergy

Mast cells are present in greater numbers, are in a state of continuous degranulation and are in a heightened state of activation in allergic disease such as asthma and allergic rhinitis (Flint et al., 1985; Kirby et al., 1987; Beasley et al., 1989; Godard et al., 1987; Bentley et al., 1992; Enerback et al., 1986). Mast cell mediators which have many important pro-allergic functions, such as histamine, tryptase, LTC4 and PGD2,

are found in high levels and are thought to co-ordinate the EPR (Kirby et al., 1987; Casale et al., 1987; Wenzel et al., 1988; Wardlaw et al., 1989).

The ability of mast cells to synthesise, store and release cytokines with important pro-allergic roles (e.g. IL-4 and IL-5) has implicated mast cells as co-ordinators of allergic inflammation. Mast cells in allergic disease have been shown to produce such cytokine, and to constitute a high percentage of cells containing such cytokine (Ying et al., 1994; Bradding et al., 1992; 1993; 1994). Evidence suggests that such mast cell cytokine may co-ordinate the LPR and therefore perhaps the establishment of chronic allergic inflammation (Wershil et al., 1991; Howarth et al., 1991; Anderson, 1996; Goldstein, 1994; Galli, 1993).

1.6 Eosinophils and allergy

1.6.1 Eosinophils

Eosinophils are bone marrow-derived granulocytes with a bilobed nucleus and contain characteristic specific granules, as well as primary granules and small granules (Dvorak, 1994). IL-3, IL-5 and GM-CSF are important in eosinophilopoesis and the prolongation of mature eosinophil survival (see above).

1.6.2 Granule contents (preformed mediators)

Specific granules contain the basic eosinophil granule proteins (Dvorak, 1994). Major basic protein (MBP) constitutes the core of the specific granule and represents 50% of the total granule protein (Gleich and Adolphson, 1986). It is a 117 amino acid single polypeptide chain with a molecular weight of 14,000 and its arginine-richness accounts for its basicity (Gleich and Adolphson, 1986). MBP has no enzymatic activity but is toxic to helminths, protozoa, bacteria, tumour cells and host cells. It is also able to activate neutrophils and platelets and causes non-cytotoxic release of histamine from mast cells, and other actions include non-specific complement activation and induction of bronchospasm in animal lungs (Gleich and Adolphson, 1986; Weller, 1991; Wardlaw and Kay, 1987; Jones, 1993). Its toxic actions may stem from disruption of cell membranes (Weller, 1994).

The other granule proteins are found in the specific granule matrix (Gleich and Adolphson, 1986). Eosinophil cationic protein (ECP) is a 133 amino acid protein with a molecular weight between 18,000 and 21,000 (Gleich and Adolphson, 1986). It has ribonuclease activity and shows homology with eosinophil-derived neurotoxin (EDN) and pancreatic ribonuclease. ECP is toxic to bacteria, helminths, protozoa, host cells, is a potent neurotoxin and it exerts its toxicity by forming membrane pores (Gleich and

Adolphson, 1986; Weller, 1991; Wardlaw and Kay, 1987; Jones, 1993; Young et al., 1986). ECP also can stimulate histamine release from mast cells, stimulate fibroblasts to produce glycosaminoglycans and inhibits T cell and B cell proliferation via non-cytotoxic actions (Gleich and Adolphson, 1986; Wardlaw and Kay, 1987; Jones, 1993). EDN is a 104 amino acid polypeptide with a molecular weight of 18,000 and is a potent ribonuclease and neurotoxin but is only weakly toxic to parasites and host cells (Gleich and Adolphson, 1986; Jones, 1993; Weller, 1991). Eosinophil peroxidase (EPO) is a cationic toxin but in the presence of peroxide and halide is more effective in killing bacteria, parasites, viruses and tumour cells (Gleich and Adolphson, 1986; Jones, 1993). It also has other effects including the non-cytotoxic release of histamine from mast cells.

Charcot-Leyden crystal (CLC) protein, which has a molecular weight of 17,000, comprises up to 10% of eosinophil protein and is found in the cell membrane and in primary granules (Gleich and Adolphson, 1986; Jones, 1993; Weller, 1991). It is a lysophospholipase and therefore acts as a surfactant (Gleich et al., 1993). Other granule contents and preformed mediators in eosinophils include collagenases, arylsulphatase, histaminase, β-glucuronidase, cathepsin, acid phosphatase, catalase, phospholipases and non-specific esterases (Spry, 1988; Kroeger et al., 1992).

1.6.3 Newly-synthesised eosinophil mediators

Upon degranulation, eosinophils synthesise a range of lipid mediators, including PAF (which has a wide range of actions including stimulation of platelet aggregation and activation, eosinophil chemotaxis, activation and degranulation and activation of other leukocytes such as neutrophils and mast cells), LTs (most prominently LTC_4, also LTB_4), PGs (PGE_2, PGD_2, PGF_2) and TxB_2, all with many proinflammatory actions (Weller, 1993). Eosinophils also produce oxygen metabolites such as superoxide anion, hydroxyl radicals, peroxide and singlet oxygen, which are important in killing micro-organisms and tumour cells but are also toxic to host cells (Petreccia et al., 1987; Kanofsky et al., 1988).

Eosinophils also synthesise cytokines (see below).

1.6.4 Eosinophil cell surface antigens and receptors

Eosinophils possess a number of immunoglobulin receptors: FCγRII (CD32, the low affinity receptor for IgG), FCεRII (CD23, the low affinity receptor for IgE), FCαR and FCδR; FCγRII (CD64, high affinity IgG receptor) and FCγRIII (CD16, low affinity) are inducible by IFN-γ (Weller, 1991; Wardlaw, 1994; Kay, 1991; Hartnell et al.,

1992). Binding of immunoglobulin to Ig receptors (especially IgA) stimulates eosinophil degranulation (Weller, 1991).

Eosinophils also bear receptors for complement (e.g. for C5a, C3b), for lipid mediators (PAF, LTs and PGs), cytokines (including IL-2 [inducible], IL-3, IL-4, IL-5, IL-8, GM-CSF, IFN-γ, -β, -α, TNF-α, RANTES, MCP-3, MIP-1α, eotaxin, LCF) and others (e.g. glucocorticoid, β-adrenoceptor and oestrogen receptors) (Weller, 1991; Gleich et al., 1993; Kroegel et al., 1992). Due to the presence of such receptors, eosinophil chemotaxis, activation, degranulation, mediator production and adhesion are influenced by many cytokines and mediators (Table 1.2). Many of these mediators are not specific for eosinophils, and also act upon other leukocytes, such as neutrophils. Because of the selective recruitment of eosinophils in allergic disease, there has been interest in mediators that show more specificity for eosinophils. Such mediators include RANTES, MIP-1α, IL-5 and LCF as chemoattractants, IL-3 and IL-4 (indirectly, by inducing VCAM-1 on endothelium) in eosinophil adherence, and IL-3 and IL-5 in eosinophil activation (Wardlaw, 1994; Nourshargh, 1993; Resnick and Weller, 1993).

A number of adhesion molecules may be expressed by eosinophils, including CD11a (LFA-1a), CD11b (MAC-1a), CD11c (P150/95α), CD18, VLA-4α (CD49d), L-selectin, ligands for E- and P-selectins, PECAM (platelet endothelial cell adhesion molecule) and SiLew X (Sialyl-Lewis X), which mediate different aspects of eosinophil adherence and emigration (Table 1.3) (Nourshargh, 1993; Resnick and Weller, 1993; Wardlaw, 1994). The VLA-4 / VCAM-1 interaction is thought to be particularly important in selective eosinophil recruitment (Resnick and Weller, 1993). Other surface antigens that may be seen on eosinophils are CD4, HLA-DR, CD9, CD69 and CD45 (Weller, 1993; Wardlaw, 1994; Gleich et al., 1993). Certain antigens, including CD4, ICAM-1, IL-2R and HLA-DR, are induced under certain conditions, such as eosinophil activation by cytokine (Weller, 1991; Wardlaw, 1994; Gleich et al., 1993; Weller, 1992).

1.6.5 Eosinophil activation

In normal individuals, eosinophils are in a resting state. Exposure of eosinophils to eosinophil-active mediators causes a priming of eosinophils, with enhancement of certain effector functions, such as chemotaxis, adhesion and cytotoxicity, and greater responsiveness to inflammatory mediators (Gleich et al., 1993; Jones, 1993; Wardlaw, 1994; Weller, 1991). Such eosinophils show greater survival, are more metabolically active, show greater expression of surface receptors (e.g. Ig receptors, complement

receptors and adhesion molecules) and expression of other surface antigens is induced (e.g. CD4, ICAM-1, IL-2R, HLA-DR, CD69, FCγRI and FCγRIII). Activated eosinophils convert granule proteins from storage to secretory forms, show enhanced degranulation upon stimulation and can produce more leukotrienes and superoxide anions; they demonstrate increased cytotoxicity, and chemotaxis to mediators such as PAF or LTB_4 is increased. In eosinophilic diseases such as asthma, allergic rhinitis and hypereosinophilic syndrome, there is a shift towards a less dense (hypodense) eosinophil phenotype, and such eosinophils show certain morphologic changes such as vacuolation and smaller-sized specific granules with loss of the granule core (Bass et al., 1980; Peters et al., 1988). Many authors believe that hypodense eosinophils represent the morphological change of activation (Weller, 1991; Wardlaw, 1994), but the published relationship of hypodensity to functional activation status has been variable and apparently partially dependent on experimental design (Prin et al., 1986; Hartnell et al., 1990).

1.6.6 Eosinophils, allergy and cytokine production

1.6.6.1 Eosinophils are central to allergy

It is well established that tissue (and sometimes blood) eosinophilia is a hallmark of allergic diseases such as asthma, allergic rhinitis and AD. Disease is associated with the presence of eosinophils and eosinophil-derived granule proteins in tissue, the blood, mucosal secretions and lavage fluids (Frigas et al., 1981; Dor et al., 1984; Kroegel et al., 1991; Frigas and Gleich, 1986; Uehara et al., 1990; Wassom et al., 1981; Leiferman et al., 1985; Bascom et al., 1989; Bentley et al., 1992). Blood eosinophils show an activated phenotype (Fukuda et al., 1985; Frick et al., 1989; Bass et al., 1980). The levels of eosinophils and their products can be correlated with direct and indirect measures of disease severity (e.g. clinical severity, bronchial hyperresponsiveness and lung function values in asthma) (Taylor and Luksza, 1987; Durham and Kay, 1985; Horn et al., 1975; Walker et al., 1993; Kagi et al., 1992). The eosinophils are believed to mediate epithelial damage in asthma, via their granule proteins (Gleich, 1990; Gleich et al., 1988; Kroegel et al., 1992). Deposition of eosinophil granule proteins is observed at areas of epithelial damage and MBP, ECP and EPO can all cause impaired function, damage and destruction of airway epithelial cells (Filley et al., 1982; Azzawi et al., 1990; Frigas et al., 1980; Motojima et al., 1989).

1.6.6.2 Eosinophils can produce cytokines

Eosinophils are capable of storage and release of cytokines, which contradicts the conventional view of the eosinophil as a simple effector leukocyte (Venge and

Hakansson, 1991). 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) (Hamid et al., 1992b; Melani et al., 1993; Yousefi et al., 1995; Costa et al., 1993; Walz et al., 1994; Lim et al., 1995) or from patients with hypereosinophilia (IL-5, IL-6, TNF-α, TGF-α, TGF-β and MIP-1α) (Melani et al., 1993; Costa et al., 1993; Desremaux et al., 1992; Dubucquoi et al., 1994; Wong et al., 1990; Wong et al., 1991; Weller et al., 1993). In atopic disease, blood eosinophils express IL-5, increased levels of IL-8 and can be stimulated to synthesise IL-3 (Yousefi et al., 1995; Tanaka et al., 1994; Fujisawa et al., 1994).

1.6.6.3 Eosinophil cytokine production in allergy and disease

Tissue eosinophils have been shown to produce cytokines in disease, including allergic disorders. In nasal allergy and polyposis, IL-4, IL-5, TNF-α, TGF-α, TGF-β, and MIP-1α mRNA or protein have been co-localised to eosinophils (Costa et al., 1993; Ying et al., 1993; Saito et al., 1994; Kay et al., 1995; Howarth et al., 1995; Finotto et al., 1994; Elovic et al., 1994; Ohno et al., 1992). In asthma, BAL eosinophils contain IL-5 mRNA (Broide et al., 1992), mucosal eosinophils contain IL-5 protein (Bradding et al., 1994) and IL-4 mRNA (Kay et al., 1995) and in AD eosinophils contain IL-5 mRNA (Tanaka et al., 1994). Other disorders in which eosinophil cytokines have been identified include coeliac disease (IL-5) (Desremeaux et al., 1992), eosinophilic cystitis (IL-5) (Dubucquoi et al., 1994), dermatitis herpetiformis (IL-5) (Desremeaux et al., 1995), necrotising enterocolitis (TNF-α) (Tan et al., 1993), colonic adenocarcinoma and oral squamous cell carcinoma (TGF-α) (Wong et al., 1990), Hodgkin's disease (TGF-β) (Kadin et al., 1993) and pemphigoid (IL-10, IFN-γ) (Lamkhioued et al., 1995).

Eosinophils may therefore be involved in amplifying and orchestrating the allergic response by secretion of pro-allergic cytokines such as IL-3, IL-4, IL-5 and may also be involved in tissue repair functions through the release of TGF-α and -β (Moqbel et al., 1994) in addition to the effects mediated through eicosanoids and other mediators. The relationship between eosinophils and other cytokine-producing cells in allergy (T cells, mast cells) appears complex, although eosinophils themselves are likely to be regulated by cytokines such as IL-3, IL-5 and GM-CSF released from T cells (Moqbel et al., 1994).

1.7 Epithelial cells in allergy

1.7.1 Overview

It is now becoming clear that mucosal epithelial cells have attributes beyond that of a mere barrier and epithelial cells may be viewed as active participants in the regulation of inflammation and leukocyte behaviour. Epithelial cells or cell lines from a number of mucosal sites have been shown to be capable of functions such as the expression of cell adhesion molecules important for leukocyte emigration into tissue, the expression of MHC class II molecules important in antigen presentation and activation of T lymphocytes, the synthesis and release of PGs, LTs and HETEs which attract and activate leukocytes, and the synthesis of a variety of pro-inflammatory cytokines (Raeburn and Webber, 1994; Campbell et al., 1994; Levine, 1995; Stadnyk, 1994).

1.7.2 Adhesion molecule expression

ICAM-1 is a member of the immunoglobulin superfamily of adhesion molecules and is the ligand of LFA-1, which is widely expressed on leukocytes (Smith et al., 1993). It is expressed on vascular endothelial cells and can also be expressed by other cells including leukocytes, dendritic cells, and fibroblasts (Rothlein et al., 1986; Makgoba et al., 1988; Vogetseder et al., 1989). It is important in leukocyte-tissue cell interactions and involved in adherence of leukocytes to, and emigration of leukocytes through, vascular endothelium (Smith, 1993). Expression is increased on most cell types by cytokines IL-1, TNF and IFN-γ (Dustin et al., 1986; Pober et al., 1986). Nasal and bronchial epithelial cells are capable of expressing ICAM-1 and this expression is markedly increased in allergic rhinitis and asthma, correlating with clinical severity (Vignola et al., 1993; Gosset et al., 1995; Montefort et al., 1992). In rats, allergen-induced bronchial hyperresponsiveness is inhibited by pre-treatment with anti-ICAM-1 antibodies, suggesting that epithelial cell expression of ICAM-1 does lead to cell interactions with important effects upon disease (Sun et al., 1994).

1.7.3 HLA-DR expression

Antigenic stimulation of T lymphocytes requires presentation of antigen in conjunction with class II MHC molecules such as HLA-DR. Human airway epithelial cells may express this molecule and it is significantly upregulated in bronchial and nasal epithelial cells in asthma and nasal polyps (Vignola et al., 1993; Stoop et al., 1989). Intestinal epithelial cells expressing class II MHC molecules have been shown capable of *in vitro* antigen presentation, preferentially stimulating $CD8^+$ T suppressor cells (Brandtzaeg et al., 1989; Bland and Warren, 1986). Whether $HLA\text{-}DR^+$ airway epithelial cells from the airway are able to present antigen is unknown.

1.7.4 Chemical mediators

Epithelial cells are able, on appropriate stimulation, to synthesise and release lipid mediators such as PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, LTB_4, LTC_4, LTD_4, LTE_4, HETEs, TxA_2 and TxB_2, and PAF (Eling et al., 1986; McKinnon et al., 1993; Adler et al., 1990; Wu et al., 1993a & 1993 b; Wright et al., 1994; Raeburn and Webber, 1994) which are major mediators of allergic inflammation, involved in alteration of vascular tone and permeability, leukocyte chemotaxis and activation, mucin production and, of relevance in asthma, bronchial smooth muscle tone. The epithelial production of such lipid mediators is under the control of cytokines (Hoeck et al., 1993; Wu et al., 1993 a & 1993b). These lipid mediators derived from epithelial cells therefore interact intimately with lipid and other mediators (e.g. histamine) and cytokines from other sources to create a complex network of interactions in the allergic inflammatory response.

The airway epithelial cells are also believed to release factors which modulate smooth muscle tone and perturbations in a balance of epithelial-derived relaxing factor(s) (EpDRF) and epithelial-derived contracting factor(s) (EpDCF), through alteration and loss of epithelial cells, may underlie the bronchial hyperresponsiveness and constriction seen in asthma and also responses to certain therapeutic agents (Bertrand and Tschirart, 1993; Raeburn, 1990; Morrison et al., 1993). It is uncertain as to exactly what these factors are; EpDRF may involve PGs and EpDCF may involve LTs, neuropeptides and endothelin-1 (Prie et al., 1990; Bertrand and Tschirart, 1993; Vittori et al., 1992). Bronchial epithelial cells are also able to produce nitric oxide (NO) which is believed to be an important mediator in asthma (Hamid et al., 1993).

1.7.5 Cytokines

Studies of epithelial cells from a number of tissues and disorders have shown that they are capable of synthesising cytokines (Raeburn and Webber, 1994; Levine, 1995; Stadnyk, 1994). Thymic epithelial cells produce an array of cytokines necessary for sustaining local lymphocytes, and other epithelial cells produce cytokines which may have both similar paracrine actions and autocrine actions (Stadnyk, 1994). Human intestinal epithelial cells can produce a wide array of cytokines including TGF-α and -β, TNF-α, IL-1, IL-8, IL-6, IL-10 and GM-CSF (Jung et al., 1995; Eckmann et al., 1993; Babyatsky et al., 1996) and other epithelial cells, such as those of the renal proximal tubule and the endometrium, have also been shown capable of cytokine production (Geritsma et al., 1996; Giacomini et al., 1995). The most detailed investigations have been conducted on airway epithelium. Human nasal and bronchial epithelial cells are capable of synthesising a wide number of cytokines, including IL-1, IL-3, IL-6, IL-8, GM-CSF, RANTES and TNF-α, MCP-1, GRO-α and GRO-γ, which

possess potent proinflammatory actions (Raeburn and Webber, 1994; Levine, 1995). This cytokine production is upregulated in epithelial cells from those with allergic respiratory and nasal disorders (Raeburn and Webber, 1994; Levine, 1995; Devalia and Davies, 1993) and may be reduced by anti-allergic therapy (Kwon et al., 1995; Davies et al., 1995; Devalia et al., 1995).

1.8 Ocular allergic disorders

1.8.1 Seasonal Allergic Conjunctivitis

Seasonal allergic conjunctivitis (SAC), the most common ocular allergy, is a recurrent conjunctivitis seen during the pollen season (Table 1.5). It occurs mainly in children and young adults sensitised to tree, grass or weed pollen. These aero-allergens dissolve in tears, diffuse into the conjunctiva and probably initiate an immune response by cross-linking mast cell-bound specific IgE to cause degranulation (type I hypersensitivity).

Patients present with seasonally-recurring itching, burning and redness of the eyes, increased tearing and sometimes a mild discharge. A personal or family history of atopy is common and there will often be an associated seasonal rhinitis. Symptoms correlate with allergen exposure and may vary daily with the pollen-count, the surroundings of the patients (e.g. rural or urban); warm, dry weather also tends to worsen symptoms. Signs range from minimal to moderate (Fig. 1.7) and are usually bilateral. Lid oedema is occasionally dramatic and patients may have an "allergic shiner", a transient increase in periorbital pigmentation due to sluggish venous return (Friedlander, 1993). Slight conjunctival hyperaemia and oedema causes a milky or pinkish appearance, but there is occasionally marked chemosis. Examination of the tarsal conjunctiva reveals a diffuse micropapillary reaction (papillae <0.3 mm diameter). The cornea is not affected and there is no conjunctival scarring. Ocular examination outside the pollen season is completely normal. The disease carries an excellent prognosis with a tendency to lessen and resolve with increasing age.

1.8.2 Perennial allergic conjunctivitis

Perennial allergic conjunctivitis (PAC) is very similar to SAC except that it is present all year round. It is poorly described in the literature and is often grouped indiscriminately with SAC. PAC is seen fairly frequently in general practice; it occurs in the young, usually starting in the early teens (Dart et al., 1986). Over two-thirds of the patients are atopic and the mechanisms involved are considered to be broadly the same as in SAC, with a type I hypersensitivity reaction initiating the inflammatory events. House dust mite (HDM, Dermatophagoides pteronyssinus) is the most

common sensitising allergen, although moulds, animal dander and other antigens may be responsible (Donshik, 1988).

Patients complain of perennial ocular itching, burning and discomfort, watering and redness.. There may be a slight mucoid discharge and mild to moderate eyelid swelling. Approximately 79% have some seasonal exacerbation and it may be possible from the history to correlate an increase in symptomatology with exposure to a particular allergen. Approximately one third have perennial rhinitis (Dart et al., 1986).

In general, PAC is less severe than SAC, and the clinical signs may be negligible. The lids may show some oedema, the bulbar conjunctiva may be injected and hyperaemia or micropapillae may be seen in the tarsal conjunctiva (Fig. 1.8). There is no corneal disease or conjunctival scarring and the prognosis is as good as SAC.

1.8.3 Vernal keratoconjunctivitis

1.8.3.1 Epidemiology

Vernal keratoconjunctivitis (VKC) is an unusual, serious ocular allergy of childhood. It represents approximately 0.1-0.5% of ocular disease and it usually affects those between the ages of 3-25 (Buckley, 1988a; Allansmith and Ross, 1988a). In 80% the onset occurs before 14 years and 85% are male (Allansmith and Ross, 1988a; Buckley, 1988a). The disease is more common in hot, dry countries particularly the Mediterranean, Middle East, West Africa, the Indian subcontinent and South America (Beigelman, 1950; Alimudden, 1955).

Approximately 75-80% of patients have a personal history and 65% a family history of atopic disease (Buckley, 1988a; Allansmith and Ross, 1988a; Frankland and Easty, 1971). Interestingly, patients from hot countries, especially the Middle East and Israel lack the association with atopy and the seasonality of symptoms (Neumann et al., 1959; Hyams et al., 1975; El-Hennawi, 1980a & b). Studies in different ethnic groups from the same locality suggest this may have a genetic rather than an environmental basis (Tuft et al., 1989; Dahan and Appel, 1983).

1.8.3.2 Clinical presentation

Symptoms are worst in the spring and summer, but in severe cases persist throughout the year and consist of itching, photophobia, blepharospasm, blurring of vision and stringy discharge. Conjunctival signs are maximal in the upper tarsal area and may be quite asymmetrical. There is hyperaemia, oedema, and infiltration of the conjunctiva with a papillary response including giant (cobblestone) papillae (diameter >1 mm) (Fig.

1.9). Reticular subepithelial scarring may be seen and tenacious mucus can make conjunctival examination difficult. At the limbus, gelatinous nodules, diffuse swelling, broadening and opacification may occur and Trantas' dots (small white focal collections of eosinophils) are seen at the tips of limbal papillae. There may be a mechanical ptosis. Patients may suffer a mainly tarsal inflammation (especially in Caucasians), a limbal form (especially black patients) or a mixed picture.

1.8.3.3 Vernal keratopathy

Keratopathy is most likely to involve the superior third of the cornea (Fig 1.10). Buckley (Buckley, 1981) has classified vernal keratopathy into several stages:

1. Punctate epithelial keratitis: discrete or coalescent, usually seen as a grey speckling of the epithelium with fluorescein staining
2. Epithelial macroerosion (a discrete epithelial defect)
3. Plaque ulcer: a shallow, round or oval "shield-like" ulcer with poor wetting by the tears and overlying mucus/epithelial debris.
4. Subepithelial scarring: ring scar, peripheral tongue-shaped scar, punctate scars
5. Pseudogerontoxon: an limited arcus-like lesion, in the shape of a crescent which waxes and wanes and is related to, but separate from, the superior limbus.

Up to one fifth of patients will suffer corneal plaque at some time (Buckley, 1981). Additional complications of VKC can include herpetic and bacterial keratitis, sensory deprivation amblyopia and problems relating to steroid treatment. Topical steroids are by far the most effective form of treatment but unfortunately have potentially sight-threatening side effects including glaucoma, cataract and the potentiation of corneal infection (Jaanus, 1989).

1.8.3.4 Prognosis

With correct management the majority will resolve spontaneously after 2-10 years or at about 16-25 years age without major corneal scarring. Occasionally, this disease metamorphoses into atopic keratoconjunctivitis in adulthood.

1.8.4 Giant Papillary Conjunctivitis

1.8.4.1 Definition and causes

Giant papillary conjunctivitis (GPC) is a foreign body-associated papillary conjunctivitis. It is uncommon and can be seen at any age and in either sex. The condition may be due to a combination of chronic low grade trauma and a hypersensitivity reaction, to the foreign material itself or, more probably, to surface biodeposits. The most common risk factor is contact lens wear, particularly soft

lenses, although it can occur with any lens type (Allansmith and Ross, 1988a;, Abelson and Allansmith, 1987). Other reported causes include ocular prostheses (Srinavas et al., 1979), exposed monofilament sutures (Nirankari et al., 1983), extruded scleral buckles and cyanoacrylate glue (Buckley, 1993). Atopy is found no more frequently than in control populations (Allansmith et al., 1977).

The probability of developing GPC during contact lens wear is increased by the following factors: long daily wear time, long-term consistent wear, larger lens diameter, soft lens wear, heavy lens deposition, lens damage (Fig. 1.11) and idiosyncratic reactivity to certain lens materials (Allansmith and Ross, 1988b). All contact lenses become deposited during wear and lysozyme, lactoferrin, albumin, PMFA (protein fraction migrating faster than albumin), IgA, IgG and protein G are all seen on the lenses of normal wearers (Lin et al., 1991). These are not completely removed by routine cleaning (Fowler and Gaertner, 1990). The condition can be alleviated by regular enzymatic protein removal or new lenses which implies that lens deposition is involved in the pathogenesis. Monkeys have been shown to mount an increased GPC-like ocular surface response to contact lenses from patients with GPC than to lenses from asymptomatic wearers (Ballow et al., 1989; Ballow et al., 1987).

1.8.4.2 Clinical presentation

Symptoms usually precede signs and the onset is very variable, ranging from 3 weeks to 5 years after beginning contact lens wear and occurring earlier in soft than hard contact lens wear (Allansmith et al., 1977). The patients complain of itching, especially on lens removal, discomfort or sometimes pain, watering, blurring of vision, reduced lens tolerance, lens decentration (usually upwards), mucus discharge and accumulation on the lens. The symptoms usually decrease with a new lens or cessation of lens wear. Clinical findings (Fig. 1.12) are most marked in the upper tarsal area. There is a papillary reaction with hyperaemia, oedema and infiltration; giant papillae (sometimes with fluorescein staining of the tips) may be seen but are not necessary for the diagnosis. Strands of mucus are seen on the conjunctiva and adherent to the lens. If severe, limbal involvement may be seen. The cornea is usually not affected, although occasionally there may be mild superior punctate fluorescein staining. The contact lens may appear deposited or may ride high.

1.8.4.3 Treatment

If the offending foreign material is removed, a dramatic improvement is usually seen within days (Allansmith et al., 1977) and the condition will resolve. This is the treatment in those with protruding sutures or explants but in the case of contact lenses

or prostheses, most patients will wish to continue wearing the device. Usually a short period without lens or prosthesis wear is recommended until the acute findings have subsided. The contact lens hygiene regimen may need to be modified; careful and thorough hygiene is essential and cleaning/soaking solutions containing preservatives should be avoided where possible. Regular and frequent protein removal will also help. Alterations to the lens/prosthesis itself can be beneficial, such as providing a new (non-deposited) lens, improving the fit, improving the surface quality (e.g. by polishing), changing the edge profile, reducing the diameter or changing the material of the lens (e.g. change soft to rigid lenses). The wearing time may need to be reduced temporarily or permanently.

1.8.4.4 Prognosis

GPC is a completely reversible condition provided the provoking factor can be removed. With appropriate modifications of the contact lens or prosthesis, their care and wearing schedules and, in some cases, topical therapy, the vast majority will be able to resume satisfactory use of their device.

1.8.5 Atopic Keratoconjunctivitis

1.8.5.1 Definition, epidemiology and relationship to AD

Atopic keratoconjunctivitis (AKC) is a potentially serious chronic blepharokeratoconjunctivitis associated with systemic atopic disease (usually AD) and is the least common and the most poorly understood of the ocular allergic diseases. It was first described by Hogan in 1952 (Hogan, 1952). It is a disease of adults, with onset in the late teens and the maximum age range lying between 20 and 60 years. A male sex preference is generally quoted (Tuft et al., 1992; Jay, 1981; Buckley, 1988b) although the largest study to date did not confirm this (Foster and Calonge, 1990).

The incidence of any ocular involvement in AD is approximately 40% (Garrity and Liesegang, 1984) and this includes keratoconus, lens opacities and other manifestations apart from AKC (Garrity and Liesegang, 1984; Rich and Hanifin, 1985) (Table 1.4). It is difficult to give an accurate figure for the percentage of those with AD who have AKC or why they do so; whilst the lids are a common site for eczematous disease (Garrity and Liesegang, 1984) AKC itself is uncommon. Staphylococcal colonisation of the lid margins is a frequent finding in AD and it has been suggested that bacteria or their toxins could play an aetiological role in AKC via direct effects or through hypersensitivity reactions (Foster and Calonge, 1990). However, no significant difference could be found in the presence of staphylococci or their products, nor in the

presence of anti-staphylococcal immunity, between atopic dermatitics with and those without ocular disease (Tuft et al., 1992).

1.8.5.2 Clinical presentation

By definition, AKC has a 100% association with atopic disease and there is a family history of atopy in more than 50% of patients (Tuft et al., 1991; Jay, 1981). AKC is often referred to as the adult version of VKC (Buckley, 1988b). Symptoms may have a seasonal component (Foster and Calonge, 1990) but normally occur all year and consist of intense itching, burning, heavy mucoid discharge, tearing and blurred vision. There is usually eczema on the face and elsewhere although the disease may occur in association with asthma alone (Foster and Calonge, 1990).

The condition can be categorised into two types which usually remain distinct over time. In atopic blepharoconjunctivitis (ABC) the cornea is unaffected and in AKC there is keratopathy; however, AKC is often used in the clinical context to describe both entities. The lids show eczematous changes with thickening, induration, erythema, dryness, scaling and scratch marks (Fig. 1.13). Lid margin disease is usually present (Tuft et al., 1991); anterior lid margin disease manifests as erythema and scaling, sometime with small areas of ulceration, at the base of the lashes; posterior lid margin disease is seen as rounding and irregularity of the posterior margin, with hyperaemia, dilated blood vessels, and abnormal meibomian gland secretions and orifices. There may be marked lash abnormalities and the lid anatomy may be distorted by marginal notches, eversion of the punctum, ectropion and entropion.

The conjunctiva is hyperaemic, oedematous and shows a papillary reaction which may include giant papillae. A thick mucous exudate is seen. The limbus may be involved with infiltration, papillae and occasionally Trantas' dots (Jay, 1981). Cicatrisation of the conjunctiva also occurs, appearing clinically as subepithelial fibrotic scars on the tarsus (Fig. 1.14), symblepharon and shortened fornices.

1.8.5.3 AKC keratopathy

The corneal involvement may be extensive and progressive (Fig. 1.15). Punctate epithelial keratopathy, keratoconjunctivitis sicca and filamentary keratopathy are the mildest manifestations and may progress to epithelial macroerosion which can be persistent. Gradual neovascularisation with or without lipid infiltrate, progressive scarring and occasionally plaque may cause severe reduction in vision secondary to corneal opacification. Corneal complications include bacterial keratitis (especially staphylococcal), herpes simplex keratitis (which may be bilateral, severe and difficult to

treat, [Easty et al., 1975]) and keratoconus may be associated. One study showed reduction in visual acuity due to keratopathy in 40% of AKC patients (Tuft et al., 1991); in another, 75% of patients had developed a severe keratopathy of some kind (Foster and Calonge, 1990).

1.8.5.4 Prognosis

AKC usually does not resolve spontaneously and cannot be cured. It is potentially blinding (both due to disease and due to steroid-related complications) and due to the high frequency of steroid requirement, with its consequent risks, and the possible need for surgical intervention, the patients will require long-term specialist ophthalmological management.

1.9 Immunopathogenesis of chronic ocular allergy

1.9.1 Overview

The pathophysiology of ocular allergic disease remains poorly understood. Ocular allergic disorders are traditionally ascribed to type I hypersensitivity but this simplistic explanation can no longer be considered valid for conditions such as VKC and AKC and may not fully account for any ocular allergy apart from acute allergic conjunctivitis (Bonini et al., 1989; Bonini, 1993). The type of cellular and immunoglobulin response seen in SAC and PAC corresponds to that expected in a type I hypersensitivity response. In the other ocular allergic disorders, a role for type I hypersensitivity is suggested by the mast cell degranulation and eosinophilic infiltration seen in histological specimens (Tuft et al., 1991; Abelson et al., 1983a; Allansmith et al., 1979; Foster et al., 1991), the increased prevalence of a personal or family history of atopic disease which has often been found (Frankland and Easty, 1971; Allansmith et al., 1977; Tuft et al., 1991) and the raised total or specific IgE frequently seen in the tears or serum with a high rate of positive skin prick tests (Ballow and Mendelson, 1980; Baryishak et al., 1982; Samra et al., 1984; Kari et al., 1985a; Donshik and Ballow, 1983). However, there is evidence suggesting that other immune mechanisms are involved in GPC, VKC and AKC: the cellular infiltration is atypical of type I reactions (see below), there remains a considerable number of patients without an atopic background or high serum and tear IgE, and there may be a poor response to therapy with anti-histamines and mast cell membrane stabilisers (Bonini 1993; Ballow et al., 1983; Sompolonsky et al., 1982).

1.9.2 Methods of investigation

In contrast with other allergic disease, the study of atopic inflammation in the eye is facilitated by the accessibility of tissue. The mucosa is directly visible, tissue samples

can be easily and safely obtained, and tear samples, which are informative for mediators, immunoglobulins and inflammatory cells, can be collected. In addition, a number of experimental models of allergic conjunctivitis have been developed in animals and humans which have proved valuable in the investigation of the mechanisms of atopic eye disease (especially SAC and PAC). These include the conjunctival allergen challenge (Abelson et al., 1990a; Merayo-Lloves et al., 1996), the conjunctival histamine challenge (Abelson and Udell, 1981; Abelson and Smith, 1988), the compound 48/80 challenge (inducing pharmacological stimulation of conjunctival mast cell degranulation) (Abelson et al., 1983b; Abelson and Smith, 1988) and the "ocular anaphylaxis" model (induced by injection of antigen into the ocular adnexae of previously sensitised animals) (Allansmith et al., 1983; Allansmith et al., 1981a)

1.9.3 Immunoglobulins

Mean total serum IgE is significantly raised and a high serum IgE occurs more frequently in VKC than in normal controls (Allansmith et al., 1976, Samra et al., 1984, Easty et al., 1980) although this has not been a consistent result in all series (Ballow et al., 1983; Sompolinsky et al., 1982). Grass pollen-specific IgE is found in the serum in up to 69% of VKC cases and correspondingly 25/35 have positive skin prick tests (Allansmith and Frick, 1963) but this cannot be related to the severity of the disease (Allansmith and Frick, 1963).

In the tears, total IgE is also raised (Baryishak et al., 1982; Ballow et al., 1983; Brauringer and Centifano, 1971; Sompolinsky et al., 1982) although one study failed to confirm this (Allansmith et al., 1976). There are also raised levels of pollen-specific IgE in the tears in VKC and these are locally produced (Ballow and Mendelson, 1980; Easty et al., 1980). Other tear immunoglobulins, including total and specific IgG and total IgM, are also at higher than normal levels with local production (McClellan et al., 1973; Ballow et al., 1983). Conjunctival biopsy has shown more IgE plasma cells with higher levels of extravascular IgE in the substantia propria in VKC, but there are also increased numbers of IgA and IgD plasma cells (Allansmith et al., 1976). VKC has been reported in association with the hyperimmunoglobulin E syndrome, suggesting IgE-mediated disease; however, this syndrome is associated with other alterations in immune function, such as a severe deficiency of suppressor T cells (Butrus et al., 1984).

Although there is no increase in the frequency of atopy in GPC, the total tear IgE is raised (particularly in the worse eye) compared with contact lens wearers without GPC; IgG and IgM are also higher, but IgA is no different (Donshik and Ballow,

1983). These higher levels are due to local production. However, there is no deposition of IgE on GPC contact lenses (Richard et al., 1992) nor is there any significant difference in the degree of lens deposition of lactoferrin, lysozyme or other immunoglobulin classes except for IgM compared with non-symptomatic lens wearers (Richard et al., 1992).

The concentrations of both serum and tear total IgE are significantly raised in AKC but this is also true in those with AD and no eye disease (Tuft et al., 1991). Surprisingly, conjunctival biopsy has shown few IgE plasma cells but greater numbers of plasma cells producing other classes of immunoglobulin (Foster et al., 1991). However, plasmapheresis has been used successfully to treat a small number of AKC patients with high serum levels of IgE (Aswad et al., 1988).

1.9.4 Inflammatory mediators

1.9.4.1 Histamine

Histamine produces vasodilatation, increased post-capillary permeability, chemokinesis of granulocytes and release of neuropeptides via an antidromic reflex (Schwartz, 1988). The histamine conjunctival challenge (where histamine is applied topically to the conjunctiva) mimics typical ocular allergic signs including itching, redness and mucus production (Abelson and Udell, 1981). The reaction requires both H1 and H2 histamine receptor stimulation; itch is mediated primarily by H1 receptors and vasodilatation by H2 receptors (Abelson and Udell, 1981; Weston et al., 1981; Leon et al., 1986).

Conjunctival allergen challenge produces a rapid rise in tear histamine (Kari et al., 1985b) and increased levels of histamine are present in the tears in VKC (Abelson et al., 1977; Abelson et al., 1980a). In VKC, histaminase activity is reduced in the serum and tears (Secchi et al., 1995) which may partially explain the hyper-responsiveness to ocular histamine challenge seen in these patients (Bonini et al., 1992).

1.9.4.2 Tryptase

Tryptase can be detected in tears following conjunctival allergen challenge or after pharmacological stimulation of mast cell degranulation by topical compound 48/80, and also in active SAC, VKC and GPC (Butrus and Abelson, 1988; Butrus et al., 1990). Its role is not well defined but may involve digestion of vascular basement membrane and connective tissue components (facilitating leukocyte access), activation of growth factors and neuropeptides, modulation of the kinin, complement and fibrinogen systems, and stimulation of chemotaxis and secretion by eosinophils and

mast cells (Serafin and Austen, 1987; Schwartz, 1988; Tam and Caughey, 1990; Walls et al., 1995).

1.9.4.3 Arachidonic acid metabolites

Both direct and indirect evidence implicates arachidonic acid metabolites in atopic eye disease. In animals and man, topical arachidonic acid or PGD_2 (the major mast cell prostaglandin, Holgate, 1988) produce clinical changes resembling atopic eye diseases and an eosinophilic infiltrate in the conjunctiva (Abelson et al., 1985 & 1987) and topical LTB_4, LTD_4 and LTE_4 generate an eosinophilic conjunctival infiltrate with a potency 1000 times greater than histamine (Spada et al., 1986; Trocme et al., 1989a). Conjunctival allergen challenge produces a rise in tear LTC_4, LTD_4 and LTE_4 (not reproduced in reflex tearing) (Bisgaard et al., 1985) and there are increased levels of PGF (Dhir et al., 1979) and HETEs (Abelson, 1983) in vernal tears.

1.9.4.4 Platelet activating factor

PAF is a highly potent mediator synthesised by mast cells and other leukocytes, which has been implicated in non-ocular atopic diseases (Kroegel et al., 1992). Its actions include platelet aggregation, vasodilatation, increased vascular permeability, granulocyte chemotaxis and eosinophil activation and degranulation (Kroegel, 1992; Wardlaw et al., 1986b). The ocular surface can synthesise PAF (Thakur et al., 1996) and topical PAF produces increased vascular permeability, hyperaemia and oedema of the conjunctiva (Braquet et al., 1984; Stock et al., 1990) with an eosinophil and neutrophil infiltrate (George et al., 1990).

1.9.4.5 Complement

The complement system is an important mediator of tissue inflammation and many complement components have been identified in the ocular surface (Mondino and Phinney, 1988). C3 complement levels are raised in the tears of patients with atopic eye disease (Mondino and Phinney, 1988) and increased levels of both C3 and factor B, with local production, have been shown in VKC (Ballow et al., 1985).

1.9.5 Histopathology

1.9.5.1 VKC histopathology

In VKC, the conjunctival substantia propria is infiltrated by eosinophils, mast cells, basophils, plasma cells, lymphocytes (mainly T cells) and some neutrophils and fibroblasts and an increased number of activated macrophages are also present (Morgan, 1971; El-Asrar et al., 1989a; Allansmith et al., 1978 & 1979; Collin and Allansmith, 1977; Metz et al., 1996; Bacon et al., 1993). Large papillae are obvious,

immature lymphoid follicles occur and there is marked collagen deposition (Morgan, 1971; El-Asrar et al., 1989a; Collin and Allansmith, 1977) (Fig. 1.16). Endothelial cell swelling and death are visible in submucosal blood vessels with extravasation of red blood cells and fibrin (Collin and Allansmith, 1977).

The epithelium shows cellular proliferation with downgrowths into the substantia propria and occasional keratinisation. Epithelial cells at the tips of papillae undergo mucinous degeneration and there is an increase in the number of goblet cells in the crypts between papillae (Morgan, 1971). Inflammatory cells, including mast cells, eosinophils, basophils and plasma cells, migrate into the epithelium and there are many dendritic cells (El-Asrar et al., 1989a; Collin and Allansmith, 1977; Allansmith and Baird, 1978; Allansmith et al., 1979; Bacon et al, 1993; Metz et al., 1996). Since it is possible to see a large number of leukocytes in the normal conjunctiva, Allansmith suggested certain findings (found in VKC and, to a lesser extent, in GPC) which can always be regarded as pathological: eosinophils, basophils, mast cells and plasma cells in the epithelium; eosinophils and basophils in the substantia propria (Allansmith et al., 1979).

Mast cells in the epithelium and substantia propria of VKC conjunctiva stain for surface IgE (El-Asrar et al., 1989a) and although there are greater numbers of both MCtc and MCt mast cells, the percentage of MCt cells is markedly raised (Irani et al., 1990) Ultrastructural studies have shown extensive mast cell degranulation throughout the conjunctiva (Henriquez et al., 1981).

Histological examination of excised vernal plaque material (Rahi et al., 1985) has shown a laminated structure tightly adherent to Bowman's membrane. This is composed of a mixture of mucopolysaccharides and proteins rich in tyrosine- and sulphur-containing amino acids, immunoglobulins, complement, and young and old fibrin. Cell debris, necrotic epithelium and a few inflammatory cells are also present.

1.9.5.2 AKC histopathology

In AKC there is a dense substantia propria infiltrate consisting of mast cells, eosinophils, lymphocytes (mainly T cells, with occasional granuloma formation), macrophages, neutrophils, B cells and plasma cells, and in the epithelium, mast cells, eosinophils, T lymphocytes, macrophages and increased numbers of dendritic cells are present (Foster et al., 1991; Morgan et al., 1991b; Bacon et al., 1993; Metz et al., 1996). Goblet cell proliferation and pseudotubular formation are seen in the epithelium, and collagen deposition occurs in the substantia propria (Foster et al.,

1991). There appears to be a denser infiltrate of mast cells, eosinophils and neutrophils in AKC when the cornea is involved than when it is not (Bacon et al., 1993). In both VKC and AKC, conjunctival scrapings may reveal eosinophils or free eosinophil granules (Abelson et al., 1983a; Friedlander et al., 1984), and raised levels of eosinophil granule proteins have been measured in tears (Montan and Hage-Hamsten, 1994; Udell et al., 1981; Saiga et al., 1991).

1.9.5.3 GPC histopathology

In GPC, the conjunctiva is thrown up into large papillae with irregular epithelial thickening and extensions of epithelium into the substantia propria. There are fewer goblet cells at the papillary tips and hyperplastic goblet elements in the interpapillary crypts (Allansmith et al., 1977). The conjunctiva is infiltrated with mast cells, eosinophils, basophils, neutrophils, lymphocytes and plasma cells. There is collagen deposition in advanced disease (Allansmith et al., 1977).

The histopathological features of GPC are qualitatively similar to those in VKC but occur to a lesser degree. Conjunctival scrapings frequently show eosinophils or free eosinophil granules (Friedlander et al., 1984) and there is a significant increase in MBP deposition in the conjunctiva (Trocme et al., 1989b) although MBP deposition in the contact lens is an unusual finding (Trocme et al., 1990). The epithelial mast cells are MCtc type but there is no increase in substantia propria mast cell numbers (Irani et al., 1990). Substantia propria and epithelial mast cells are degranulated (Henriquez et al., 1981; Allansmith and Baird, 1981).

1.9.6 Adhesion molecules

Adhesion molecules are pivotal to the recruitment of inflammatory cells into tissue and differential expression is likely to determine the nature of the cellular infiltrate (Smith et al., 1993). Characteristic changes in the type and degree of adhesion molecule expression have been documented in non-ocular atopic disease for which cytokines are believed to be important regulatory factors (Smith et al., 1993).

Conjunctival allergen challenge results in an early (30 minutes) upregulation of E-selectin followed by ICAM-1 and VCAM-1 (4-24 hours) on vascular endothelial cells (Bacon et al., 1998). The vascular expression of E-selectin, ICAM-1 and VCAM-1 is upregulated in all the atopic eye diseases (greatest in VKC) and correlates with the degree of cellular infiltrate (Bacon et al., 1998; Saiga et al., 1994; Tabbara et al., 1996). The VLA-4 / VCAM-1 interaction is believed to be crucial for selective eosinophil recruitment in atopic disease (Resnick and Weller, 1993) and, in atopic eye

disease, vascular VCAM-1 correlates the most closely with eosinophil numbers (Bacon et al., 1998).

1.9.7 Mast cells in ocular allergy

There is much evidence to suggest that mast cells and their mediators are important in the pathogenesis of atopic eye diseases (see above). In the normal human conjunctiva, mast cells are found only in the substantia propria and the vast majority are MCtc (Irani et al., 1990; Baddeley et al., 1995). In atopic eye disease, mast cells are found in the epithelium and there is an increase in the proportion of MCt, particularly in the epithelium, although MCtc remains the most common phenotype (Irani et al., 1990). The degree of these changes, and subtle variations occurring in the expression of other proteases (e.g. carboxypeptidase A, cathepsin G), differ in each clinical syndrome (Baddeley et al., 1995). As mast cell subtypes have different pharmacological characteristics (Holgate, 1991), such inter-disease variation may have important implications for the response to drug therapy.

Mast cells are capable of synthesising, releasing and, unlike T cells, storing pro-allergic cytokines and this has been implicated in the pathogenesis of non-ocular mucosal atopic disease (Bradding et al., 1993). Conjunctival mast cells have been shown to contain IL-4, IL-5, IL-6, and TNF-α cytokine protein in normal and SAC patients and after conjunctival allergen challenge (MacLeod et al., 1995 & 1996; Anderson, 1996). IL-8 is present only after allergen challenge and in SAC (MacLeod et al., 1995), and there is upregulation of mast cell IL-4 during the pollen season in SAC (MacLeod et al., 1996). It has been suggested that release of stored mast cell cytokine may orchestrate the early allergic inflammatory response and may provide a link between this initial response to allergen and chronic atopic inflammation through stimulation of T cells and perhaps promotion of Th2 differentiation (Goldstein, 1994; Anderson, 1996).

1.9.8 T cells in ocular allergy

1.9.8.1 Evidence for T cell inflammation in chronic ocular allergy

In VKC, AKC and GPC, T lymphocytes are the predominant infiltrating cell type (Metz et al., 1996; Foster et al., 1991). In the normal conjunctiva, $CD8^+$ cells outnumber $CD4^+$ cells in the epithelium with a higher CD4:CD8 ratio in the substantia propria where CD4 cells may slightly outnumber CD8 cells (see above). In VKC, AKC and GPC, both $CD4^+$ and $CD8^+$ T cells increase in number, but $CD4^+$ T cells predominate in all conjunctival layers (Metz et al., 1996; Foster et al., 1991; Bhan et al., 1982). T cells seem to be activated with increased T cell expression of IL-2

receptor (CD25)and HLA-DR in AKC, VKC and GPC (Foster et al., 1991; Metz et al., 1996; Bonini et al., 1995), and there are increased numbers of memory ($CD45Ro^+$) T cells in these disorders (Metz et al., 1996).

1.9.8.2 T cell cytokines and Th2-like cells in ocular allergy

In view of the apparent importance of eosinophils and mast cell-IgE mechanisms in atopic eye disease, it has been suggested that cytokines which promote IgE synthesis and stimulate eosinophils are likely to play an important role (Bonini et al., 1995). It is hypothesised that these cytokines (IL-3, IL-4, IL-5, GM-CSF, RANTES etc.) are mainly derived from Th2-like T cells; this is widely believed to underlie the pathogenesis of other atopic diseases (Bonini et al., 1995; Maggi and Romagnani, 1994; see above).

Previous work suggests a role for these cytokines in atopic eye disease. In transgenic mice, over-expression of IL-4 resulted in a blepharoconjunctivitis with histology reminiscent of allergic inflammation and a raised serum IgE (Tepper et al., 1990). Elevated levels of serum IL-4 occur in children with SAC (Ohshima et al., 1995) and of tear IL-4 in patients with SAC and VKC (Fujishima et al., 1995). Tear IL-5 is increased in VKC (Bonini et al., 1995) and tear RANTES in VKC, PAC and SAC (Takamura et al., 1995). However, such investigations do no identify the cellular source of cytokine, which may include mast cells and other cell types.

Peripheral blood T cells from patients with VKC have been shown to provide significantly greater help for B cell IgE production than T cells from healthy controls (Yuasa et al., 1985). Cultures of T cell clones derived from the conjunctiva of patients with VKC have shown a Th2-like profile, demonstrating increased production of IL-4 and decreased production of IFN-γ compared to normal controls, whilst the production of IL-2 was unchanged (Romagnani et al., 1991; Maggi et al., 1991). These T cells from VKC conjunctiva were better able to provide help for B cell IgE synthesis than peripheral blood T cells from the same patients (Maggi et al., 1991).

1.9.9 Eosinophils in ocular allergy

1.9.9.1 Evidence for eosinophilic inflammation in chronic ocular allergy

An eosinophil infiltrate is characteristic of ocular allergic disease and is virtually pathognomonic (Abelson and Schaefer,.1993). In VKC and AKC, conjunctival scrapings often demonstrate eosinophils or free eosinophil granules, which is considered a diagnostic test (Abelson et al., 1983a; Friedlander et al., 1984). MBP is deposited in the conjunctiva in VKC (Trocme et al., 1989b) and AKC (Porter et al.,

1993). There are raised levels of MBP and Charcot-Leyden protein in VKC tears compared with normal patients and non-atopic ocular inflammation, and levels correlate with disease severity (Udell et al., 1981). ECP levels are increased in VKC and AKC tears and again correlate with disease severity and response to treatment (Saiga et al., 1991; Secchi et al., 1995; Montan and Hage-Hamsten, 1994). Serum levels of ECP and EDN are also increased in VKC (Tomassini et al., 1994).

1.9.9.2 Eosinophils and keratopathy

A number of lines of evidence have led to the hypothesis that eosinophils are important in the pathogenesis of allergic corneal damage (Trocme et al., 1989b; Santos et al., 1994). The levels of granule proteins in tears and serum correlate with disease severity (Secchi et al., 1995; Tomassini et al., 1994) and MBP deposition is observed over de-epithelialised areas in allergic corneal disease (Trocme et al., 1993). More compelling evidence still is that extrinsic MBP will adhere to denuded corneal stroma and prevent corneal re-epithelialisation in an animal model (Trocme et al., 1994).

1.9.10 Conjunctival epithelial cells in ocular allergy

1.9.10.1 Adhesion molecules & HLA-DR

Non-allergic, inflammatory ocular surface conditions such as Sjogren's syndrome are associated with expression of ICAM-1 on conjunctival epithelial cells, whereas ICAM-1 is not observed on normal conjunctival epithelial cells (Jones et al., 1994; Vorkauf et al., 1993). Corneal epithelial cells can express ICAM-1 and this is upregulated by IL-1 or in diverse corneal inflammatory disorders (Brevdik et al., 1995; Goldberg et al., 1994). In ocular allergy, ICAM-1 has been detected on conjunctival epithelial cells after allergen challenge in out-of-season SAC and also in active VKC (Tabbara et al., 1996; Ciprandi et al., 1993a).

HLA-DR expression is also induced on conjunctival epithelial cells in non-allergic ocular inflammatory conditions (Jones et al., 1994; Baudouin et al., 1992).

1.9.10.2 Cytokines

Cytokine production by epithelial cells in the cornea has been studied in more detail than that in the conjunctiva. Normal corneal epithelial cells express IL-1β, IL-8, TGF-α and -β, platelet-derived growth factor (PDGF)-β, b-fibroblast growth factor (FGF), macrophage colony stimulating factor (M-CSF), leukaemia inhibitory factor (LIF) and insulin-like growth factor (IGF)-1 (Li and Tseng, 1995) and an IL-3-like factor (Grabner et al., 1985). Conjunctival epithelial cells have previously been shown to be a possible source for cytokines. A conjunctival cell line, "Chang", spontaneously

produces IL-1-like and IL-3-like factors (Grabner et al., 1985; Schreiner et al., 1985). Immunoreactive PDGF, bFGF, IL-2, IFN-γ and TNF-α have been demonstrated in normal and pemphigoid conjunctival epithelial cells (Bernauer et al., 1993b). Normal conjunctival epithelial cells contain mRNA for IL-1, IL-6 and IL-8 and in Sjogren's syndrome there is increased production of IL-6 compared with normal (Jones et al., 1994). There is no previously published data concerning conjunctival epithelial cytokines in ocular allergic disease.

1.10 Treatment of ocular allergy

1.10.1 Therapeutic principles

Patients with ocular allergy are often highly symptomatic, experiencing ocular itching, soreness or discomfort, redness, watering and sticky discharge and they may also have swollen lids, light sensitivity, inability to open the lids and some blurring of vision (Friedlander, 1993). In the majority of patients, who suffer from non-sight-threatening disorders (SAC, PAC, GPC), the aim of therapy is to eliminate or minimise symptoms using well-established, effective, simple and safe treatments, and it is usually possible to achieve this. A number of simple non-pharmacological manoeuvres (e.g. allergen avoidance, cold compresses) can be used to great effect. Non-specific drug therapy (e.g. ocular lubricants, mucolytics, vasoconstrictors) is helpful and specific medical treatment is with anti-histamines (topical and oral) and mast cell stabilisers (topical) (Friedlander, 1993). As a general principle, topical steroids are not indicated for the non-sight-threatening disorders due to the severity of their adverse effects. There is, however, a proportion of patients whose symptoms are not well controlled by these methods who pose a management problem.

In AKC and VKC, the aim of therapy is not only symptom reduction but also the prevention and treatment of ocular surface changes or disease complications which might reduce visual acuity, whilst avoiding iatrogenic problems. The same therapeutic modalities described above are also used in AKC and VKC. In these disorders, where there are serious potential disease complications, steroids (topical and occasionally systemic) are often used, as they are the most potent suppressors of ocular allergic inflammation. To allow the use of a minimum steroid dose, other therapy should be continued long-term and associated blepharitis and lid dermatitis should be treated appropriately. Despite this, steroid-related complications occur (Jaanus, 1989), but we lack drugs able to adequately suppress serious ocular allergic inflammation without serious side-effects or poor patient tolerability.

1.10.2 Removal of the cause

It may be possible to aid symptom control by reducing exposure to the precipitating factor. In SAC and VKC, where pollen is implicated, simple allergen avoidance consists of keeping away from grassy fields, trees and flowers, keeping windows shut and staying indoors, especially on high-pollen count days. In more serious disease, admission to hospital, tarsorrhaphy or occlusive goggles may occasionally by required (Buckley, 1988a). In house dust mite sensitivity (PAC) manoeuvres such as removal of the bedroom carpet, vacuuming the mattress, using mite-impermeable pillow and mattress covers, washing bed linen and curtains at mitocidal temperatures (>55^{o}C) and using mitocidal chemicals may help; in mould sensitivity, the use of dehumidifying devices is recommended (Christiansen, 1988).

In GPC, elimination of, or decreasing exposure time to, the causative foreign body is often indicated (Allansmith et al., 1977). Modifications to a contact lens or ocular prosthesis, including careful lens hygiene, frequent protein removal, polishing to remove surface and edge irregularities and changes to the lens shape and material, and reduced wearing time all help to relieve the condition (Allansmith and Ross, 1988a; Donshik 1988).

1.10.3 Immune manipulation

The mechanism of action of immunotherapy (desensitisation) is uncertain, and may involve induction of blocking antibodies or alteration of T cell subsets (Weins and Jackson, 1988). In SAC and seasonal rhinitis, both parenteral and oral immunotherapy have been shown to be effective in reducing symptoms and conjunctival allergen challenge reactivity, and they may help in PAC (Bjorkstein et al., 1986; Bloch-Michel, 1988; Moller et al., 1986). Since there is a potential risk and a long duration of treatment, they are reserved for particularly serious cases. Immunotherapy has not been shown to be effective in the other ocular allergies.

Plasmapheresis has been shown to be an effective treatment for severe AKC when the total serum IgE level is raised, and presumably acts by reducing the level of allergen-specific IgE (Aswad et al., 1988). Its use in other ocular allergic disease has not been explored.

1.10.4 Non-specific medical therapy

General measures such as cold compresses and obtaining a cool, moist atmosphere (e.g. air-conditioning, geographical relocation) can reduce symptoms. Conjunctival irrigation using normal saline or lubricant drops (artificial tears) aids comfort and may

help dilute the allergen and reduce allergen-conjunctiva contact time. Heavy mucoid discharge can be thinned with topical mucolytics such as acetylcysteine.

Sympathomimetic vasoconstrictors (Table 1.6), such as naphazoline and tetrahydrazoline, reduce symptoms and signs (especially hyperaemia) in conjunctival allergen challenge tests and ocular allergies (Abelson et al., 1980b & 1990). They are particularly useful in SAC and PAC and are usually prescribed in combination with a topical antihistamine. There has been concern regarding tolerance, rebound vasodilatation and permanent loss of vessel tone (Friedlander, 1991) but this is unproved (Abelson et al., 1984).

1.10.5 Mediator inhibition

1.10.5.1 Antihistamines

Topical anti-H1 histamine receptor antagonists in combination with a vasoconstrictor are in common use for symptom relief in PAC and SAC. There is a synergistic relationship between the two drug components (Abelson et al., 1980b & 1990b). Topical anti-histamines are safe. They achieve a high local concentration and lack the systemic side-effects of oral anti-histamines but, conversely, offer no treatment to associated systemic allergic manifestations, such as rhinitis. Because of their mode of action (i.e. direct inhibition of mediator action after release) they have a quick onset of action but no preventative effect, and potency is limited as they antagonise only one of the many inflammatory mediators involved in ocular allergy (Serafin and Austen, 1987). They are helpful in SAC and PAC but are of little use in AKC, GPC and VKC.

Levocabastine is a newer, topical, specific H1 receptor antagonist with a very high potency and rapid onset of action compared with other anti-histamines (Dechant and Goa 1991; Parys et al., 1992). Levocabastine is significantly more effective than conventional anti-histamine-vasoconstrictor drops in SAC and PAC and compares favourably with sodium cromoglycate, with a more rapid onset of action (Azvedo et al., 1991; Bende and Pipkorn, 1987; Davies and Mullins, 1993; Pipkorn et al., 1985; Ciprandi et al., 1990; Frostaad and Olsen, 1993; Janssens and Blockhuys, 1993; Janssens and VandenBusse, 1991; Odelram et al., 1989; Tiszler Cieslik et al., 1994). Currently, its use in the UK is limited by its licence for only 4 weeks in any one year.

Oral anti-histamines are widely used in SAC and PAC and occasionally in VKC and AKC as an adjunct (Ciprandi et al., 1992). They have the advantage of also treating rhinitis (Ciprandi et al., 1992). Their onset of action is slower, and the local conjunctival concentrations lower than for topical preparations (Howarth et al., 1984).

First generation drugs (e.g. chlorpheniramine) are associated with drowsiness and anti-muscarinic effects which limit their use (although the sedation can be an advantage for nocturnal pruritus) (Ciprandi et al., 1992). Second and third generation anti-histamines, such as terfenadine, astemizole and cetirizine, cause less sedation due to poor penetrance of the blood-brain barrier and are effective in ocular allergy (Ciprandi et al., 1992 & 1993b; Bussche et al., 1987; Schoeneich and Pecoud, 1990). However, there is an increasing awareness of serious side-effects, particularly cardiac arrhythmias, which means that they should be used with caution in ocular allergy, for which topical preparations are available (Woosley, 1996).

1.10.5.2 Cyclo-oxygenase inhibitors

A number of topical, non-steroidal anti-inflammatory drops are available, mainly for use in the prevention of intraoperative miosis and the treatment of postoperative inflammation and cystoid macular oedema. Some of these drops have been assessed in ocular allergy. Suprofen 1% drops produced better clinical improvement than placebo in VKC (Buckley et al., 1986) and GPC (Wood et al., 1988) and diclofenac 0.1% drops were superior to placebo in SAC (Laibovitz et al., 1995). Flurbiprofen 0.03% reduced symptoms and signs in PAC (Maldonaldo et al., 1996) and tolmetin 2% gave clinical improvement in VKC (Syrbopoulos et al., 1986). Ketorolac tromethamine 0.5% produced better clinical control in 2 placebo-controlled studies in SAC (Ballas et al., 1993; Tinkelman et al., 1993). Most studies have been placebo comparisons and it is not clear from the limited data (Maldonaldo et al., 1996; Syrbopoulos et al., 1986; Guzman et al., 1996) how topical cyclo-oxygenase inhibitors compare to accepted therapies. Only ketorolac is licensed specifically for use in ocular allergy (not in the UK) and an independent review concluded that limited data and high expense precluded its routine use (Anon, 1993).

Oral aspirin has been used as an adjunct in VKC and may be helpful in healing corneal damage (Abelson et al., 1983c; Srinvas, 1989) although it is contraindicated in those under eleven years of age because of the risk of Reye's syndrome. Cyclo-oxygenase drugs are not widely used in the United Kingdom for ocular allergy, and further evidence is awaited.

1.10.6 Mast cell inhibitors

Mast cell inhibitors, of which sodium cromoglycate is the prototype, are the mainstay of ocular allergic therapy. They are thought to act by inhibiting mast cell degranulation through changes in calcium fluxes (Cox, 1971; Spataro and Bosman, 1976), but they have a number of other actions, such as inhibition of eosinophil, neutrophil and

monocyte chemotaxis, activation, degranulation and cytotoxicity, and possibly direct mediator antagonism, which may be equally important (Bruijnzeel et al., 1990; Kay et al., 1987; Dahlen et al., 1989; Joseph et al., 1993).

Sodium cromoglycate is used extensively and is highly effective in all the ocular allergic disorders (Allansmith and Ross, 1986). Both 2% (UK) and 4% (USA) drops are available with little difference in efficacy (Collum et al., 1992; El Hennawi, 1983), and there is an ointment (4%) for supplemental use at night. Sodium cromoglycate is extremely safe and very well-tolerated; a mild stinging can occur after instillation (Lindsay-Miller, 1979). Nedocromil sodium is a newer, high potency, topical mast cell stabiliser which is also highly effective in most ocular allergic disorders (Hingorani and Lightman, 1995; Knottnerus et al., 1993). Despite its greater potency in mast cell stabilisation, there is little evidence to suggest a markedly better effect than sodium cromoglycate in practice, but it does offer the advantage of twice daily dosage (compared with four times daily for cromoglycate) in SAC and PAC (Alexander, 1995; Kjellman and Stevens, 1995).

Lodoxamide is another more recently launched mast cell stabiliser with a much greater (x2500) *in vitro* potency of mast cell inhibition than cromoglycate (Wiens and Jackson, 1988). Studies in SAC suggest a more rapid onset of action and less stinging than cromoglycate (Fahy et al., 1992) and it may be helpful in reversing very early corneal involvement in VKC (Santos et al., 1994; Caldwell et al., 1992). It has not been formally evaluated in PAC, AKC and GPC. As with cromoglycate both lodoxamide and nedocromil sodium appear to have excellent safety profiles (Alexander, 1995; Kjellman and Stevens, 1995; Anon, 1994).

Mast cell stabilisers may take up to one week for full effect and therefore rapid onset treatment is often commenced simultaneously for early control of active inflammation (Friedlander, 1995; Stock and Pendleton, 1993). They inhibit a very early stage in the inflammatory reaction (mast cell degranulation) and have wide-ranging effects via both mast cell inhibition and their other actions. This means that, unlike anti-histamines, they have a preventative action and they are most effective if commenced either before symptoms begin (e.g. before the pollen season) or early in the disease, and that they should be used continuously not episodically (Ciprandi et al., 1992; Stock and Pendleton, 1993). They play an important role as steroid-sparing agents in AKC and VKC.

Despite the development of the newer mast cell stabilisers, sodium cromoglycate continues to be widely used. Lodoxamide may be a useful alternative if cromoglycate stinging is a problem and perhaps in early vernal keratopathy, but its higher *in vitro* potency has not translated into a greater clinical effect. Nedocromil sodium offers a choice in SAC and PAC for a less frequent dosage and perhaps slightly greater potency than cromoglycate in difficult cases.

1.10.7 Steroids

Steroids have multiple anti-inflammatory actions and are potent inhibitors of allergic disease, but topical steroids have sight-threatening adverse effects including cataract, glaucoma, herpetic, bacterial and fungal keratitis (Jaanus, 1989). In AKC, herpetic keratitis is a particular problem (Easty et al., 1975). Various topical steroid preparations are available (e.g. dexamethasone 0.1%, prednisolone 0.5% and 1%); some (e.g. fluoromethalone) seem to have a greater surface action and reduced risk of glaucoma, which may be helpful in avoiding side-effects (Jaanus, 1989).

Steroids are usually best avoided in non-sight-threatening ocular allergy, where the severity of treatment adverse effects far outweighs that of the disease, except in GPC caused by an ocular prosthesis worn over a blind eye. Topical steroids are frequently required in VKC and AKC and contribute to the risk of visual reduction, via drug side-effects (Buckley, 1989; Foster and Calonge, 1990; Jaanus, 1989). In VKC, flare-ups of disease activity and keratopathy are often episodic and can be managed with short courses of strong topical steroids. In AKC, due to the chronicity and severity of disease, a large number of patients are on long-term topical steroid treatment. The dose should be kept as low as possible and for as short a duration as possible, and other therapies should be used simultaneously to achieve this. In severe VKC and AKC, systemic steroids are sometimes required, but the risk of systemic side-effects and the difficulty in weaning patients off treatment in the presence of systemic allergic disease mean that they should be avoided if possible.

1.10.8 Cyclosporin A

Cyclosporin A (CsA) is an immunosuppressive agent, most commonly used in organ transplantation, which specifically inhibits helper T lymphocyte proliferation, partly via inhibition of the transcription of IL-2 (Borel et al., 1996; Power et al., 1993; Holland et al., 1989). CsA also inhibits the expression of the interleukin-2 receptor (Foxwell et al., 1990; Haczku et al., 1996). It is therefore inhibitory to many T cell-dependent inflammatory mechanisms. It also has direct inhibitory effects on eosinophil activation and release of granule proteins and cytokines, and both direct and indirect inhibitory

effects on mast cell activation, cytokine and mediator release (Borel et al., 1996) which are likely to be important in the treatment of allergic inflammation.

Topical CsA 2% dissolved in maize oil has been used to treat a number of anterior segment conditions, including Sjogren's syndrome, ligneous conjunctivitis, ocular cicatricial pemphigoid, Mooren's ulcer and auto-immune corneal melting (Power et al., 1993; Holland et al., 1989 & 1993; Liegener et al., 1990; Zierhut et al., 1989). It has also been used in high-risk penetrating keratoplasty (Belin et al., 1989). It appears not to be absorbed into the systemic circulation in sufficient concentration to reach therapeutic or toxic dosages and therefore is not associated with any systemic side-effects (Secchi et al., 1990a & 1990b; Bleik and Tabbara 1991; Holland et al., 1993; Belin et al., 1989). Prolonged use of topical 2% preparations has been reported and the only serious side-effects produced are temporary lid maceration and corneal epitheliopathy (Secchi et al., 1990 a &1990b; Ben Ezra et al., 1986; Power et al., 1993; Belin et al., 1989). Topical CsA has not produced the serious complications of topical steroids.

There are several studies showing a marked beneficial effect of topical CsA in VKC, particularly in the control of keratopathy and as a steroid-sparing agent (Secchi et al., 1990a & 1990b; Bleik and Tabbara 1991; Ben Ezra et al., 1986, 1988 & 1993). The 2% drop is available from individual hospital pharmacies and is made by dissolving the systemic drug (which is very lipophilic) in maize oil, in a time- and labour-intensive process (Bleik and Tabbara, 1984; Holland et al., 1993). Use of CsA in VKC is currently limited by its availability to certain large ophthalmic centres. Systemic CsA is occasionally used for very severe, sight-threatening disease or in high-risk corneal surgery in AKC and VKC patients, but carries the risk of serious adverse effects, such as renal disease and hypertension (Borel et al., 1996).

1.11 Objectives of the project

The aim of the studies in this thesis is to clarify the immune processes underlying chronic allergic eye disease, particularly the sight-threatening complications (corneal disease), and to investigate the clinical response to, and local immune effects of, CsA drops in AKC, which is the most severe and blinding disorder. This will be achieved using methods which include conjunctival biopsy, histopathological examination of conjunctiva by tissue stains, immunohistochemistry, *in situ* hybridisation and a randomised, placebo-controlled, double-masked clinical trial.

The aim of the first part of the project is to quantify the number and examine the phenotype of the leukocyte population of the normal conjunctiva, which will provide a basis for understanding the changes found in allergic eye disease.

The second portion of the project will examine the number and phenotype of leukocytes, and their cytokine production, in chronic allergic eye disease with corneal disease (sight-threatening), chronic allergic eye disease without corneal disease (non-sight-threatening), and normal subjects. The leukocyte types which are particularly important in chronic allergy are T lymphocytes, eosinophils and mast cells. This project will focus on T lymphocytes and eosinophils, as mast cells are being investigated by Southampton University in linked research.

It is possible that conjunctival epithelial cells contribute to the inflammatory process in chronic allergic eye disease, as lung epithelial cells do in asthma. The third part of the thesis will investigate this hypothesis by examining the phenotype of, and cytokine production by, conjunctival epithelial cells in chronic allergic eye disease (with and without corneal disease) and normal controls.

CsA is an immunomodulatory drug used in organ transplantation and some systemic inflammatory disorders. The fourth part of the thesis will investigate the use of this established drug in drop form for a new indication, AKC. Patients with this condition will undergo treatment with this drug and will be assessed for clinical response, including symptoms, clinical signs and particularly in the change of steroid drop requirement, as well as for drug side-effects. Also, the leukocyte phenotype and cytokine production before and after treatment will be assessed.

Chapter 1: Tables and figures

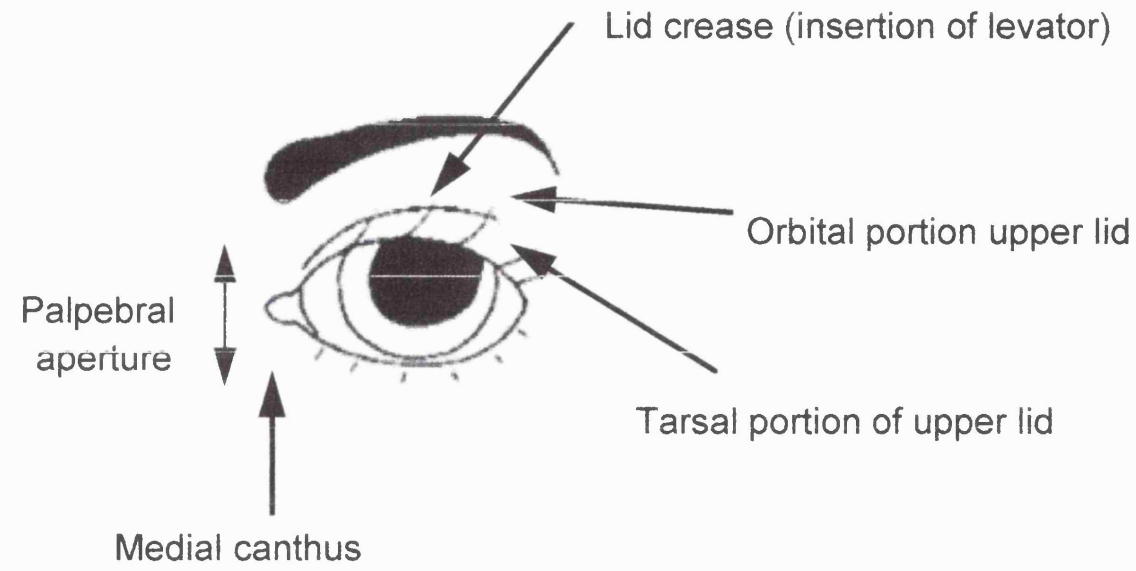


Figure 1.1 Surface anatomy of the lids

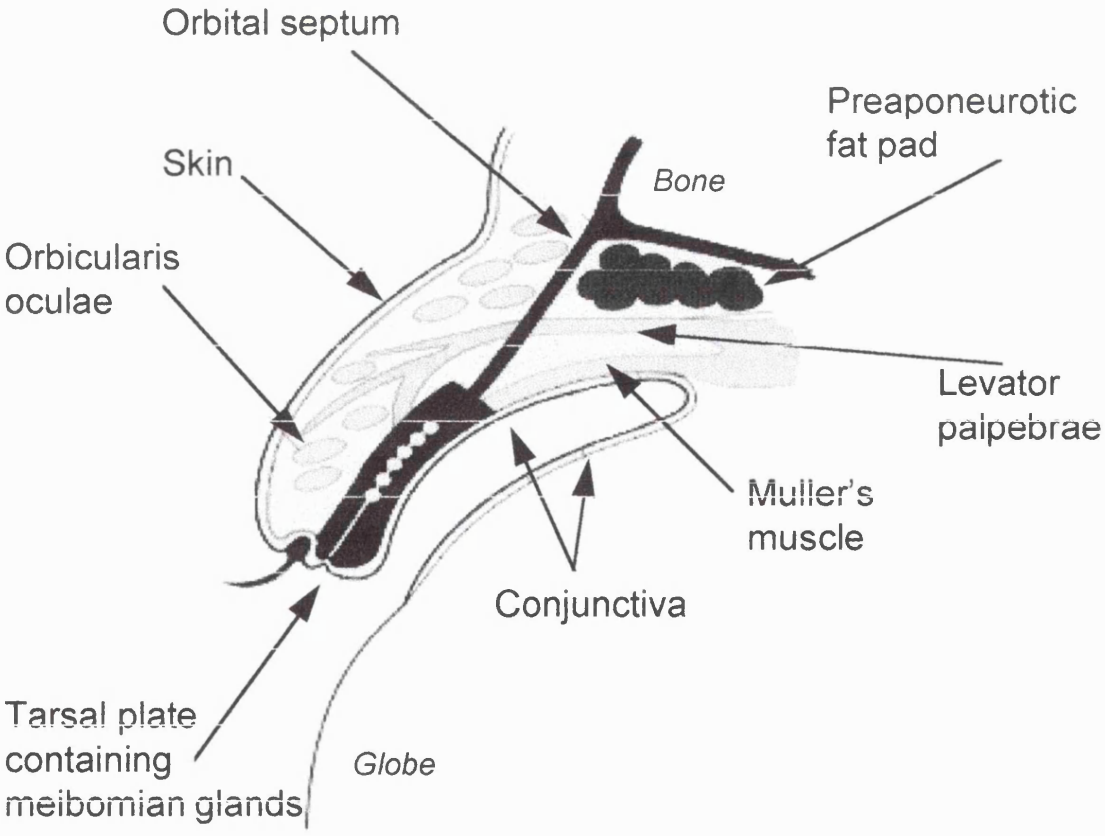


Figure 1.2. Cross-sectional anatomy of the upper eyelid

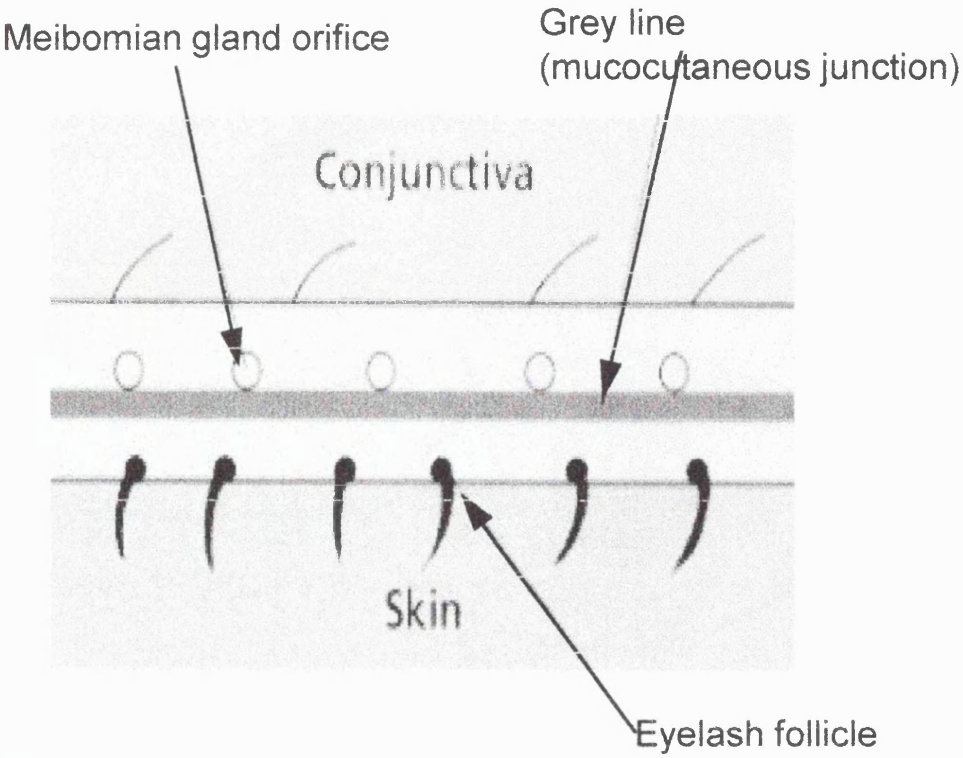


Figure 1.3a. Surface anatomy of the lid margin.

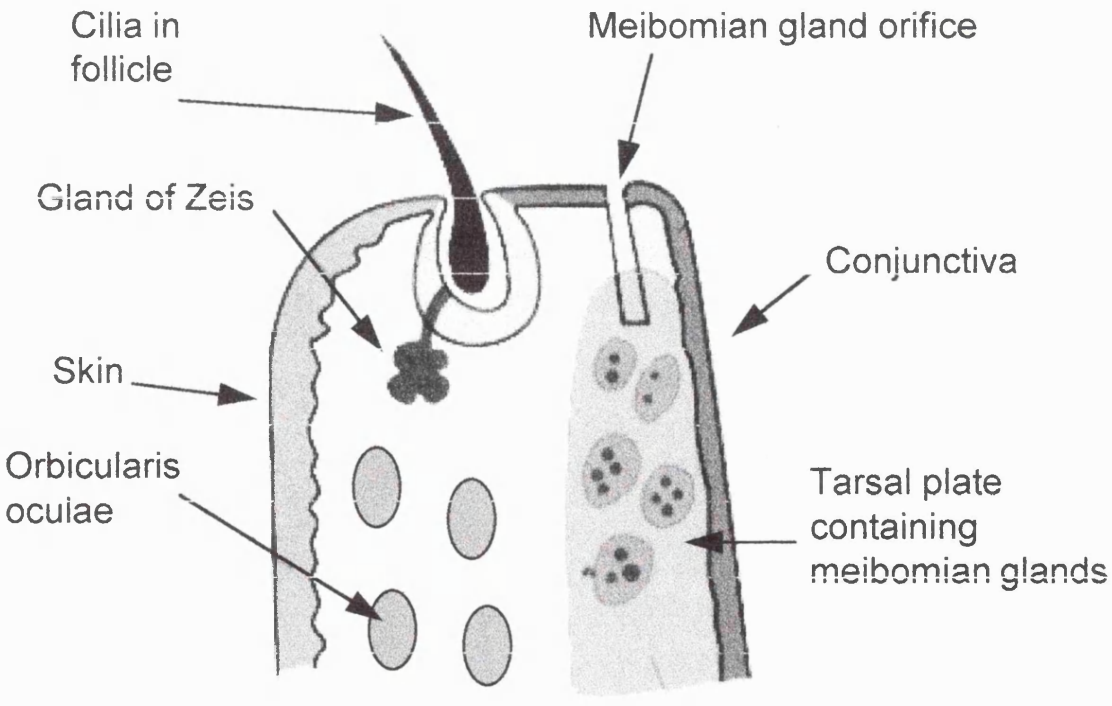


Figure 1.3b. Cross-section through the lower lid margin.

Figure 1.3. Anatomy of the lid margin.

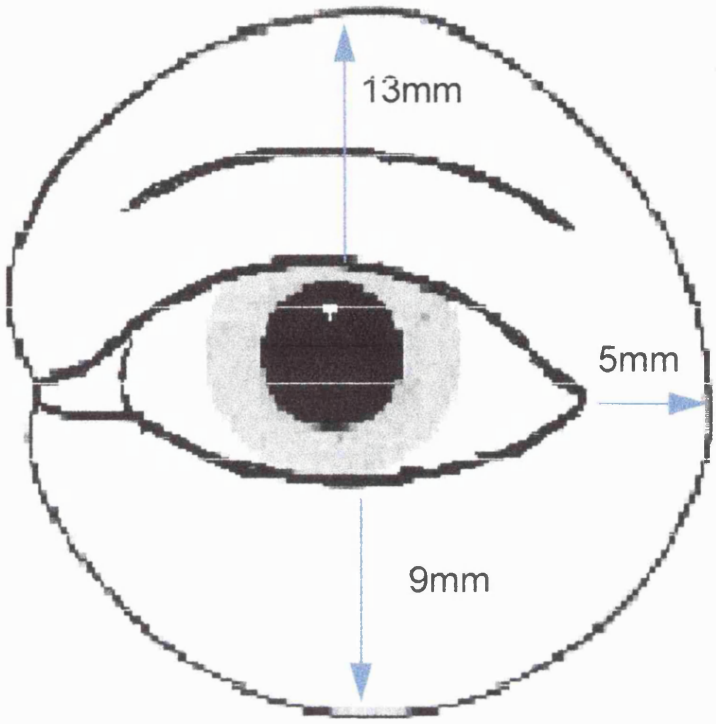


Figure 1.4. Surface markings of the conjunctiva.

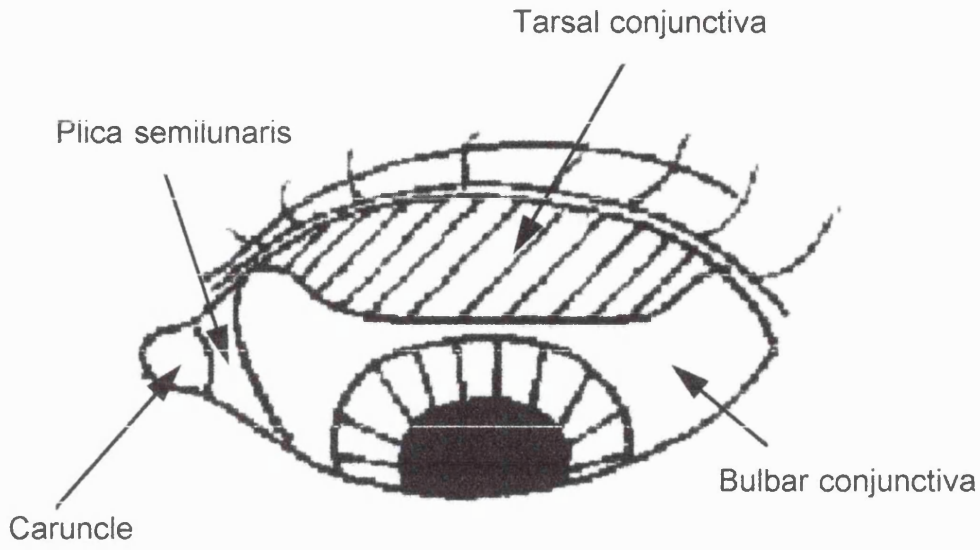


Figure 1.5a. Clinical divisions of the conjunctiva with the upper lid everted.

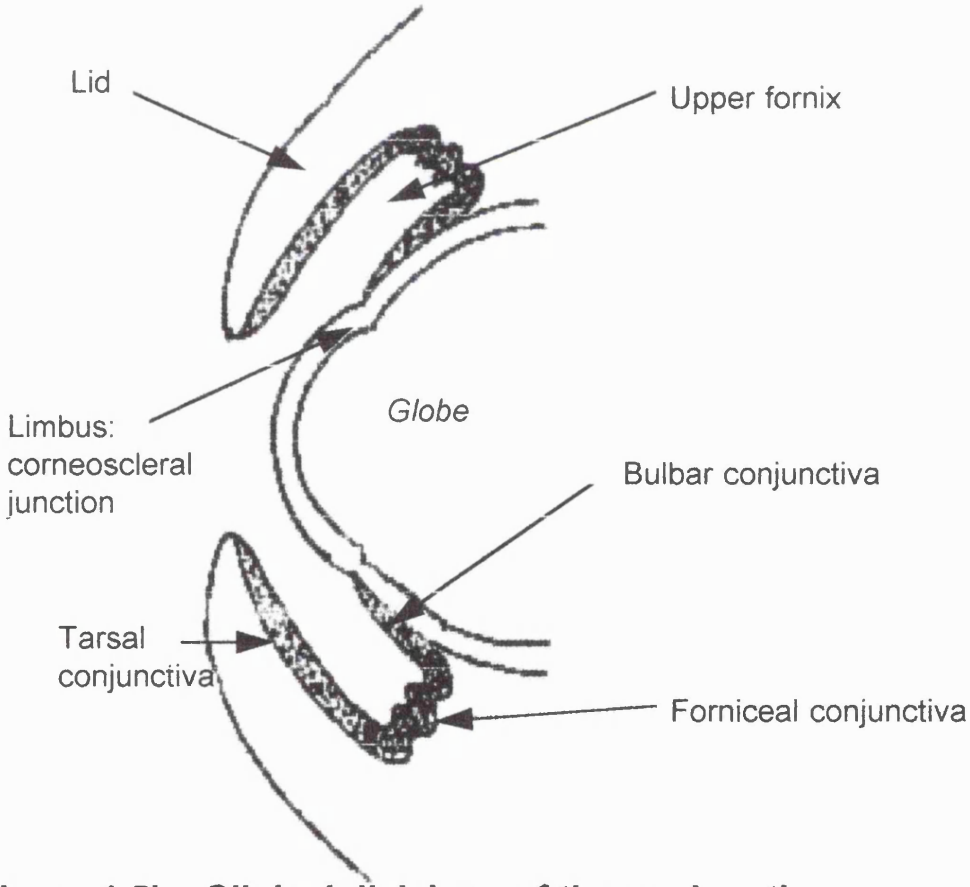


Figure 1.5b. Clinical divisions of the conjunctiva: cross-sectional view

Figure 1.5. Clinical divisions of the conjunctiva

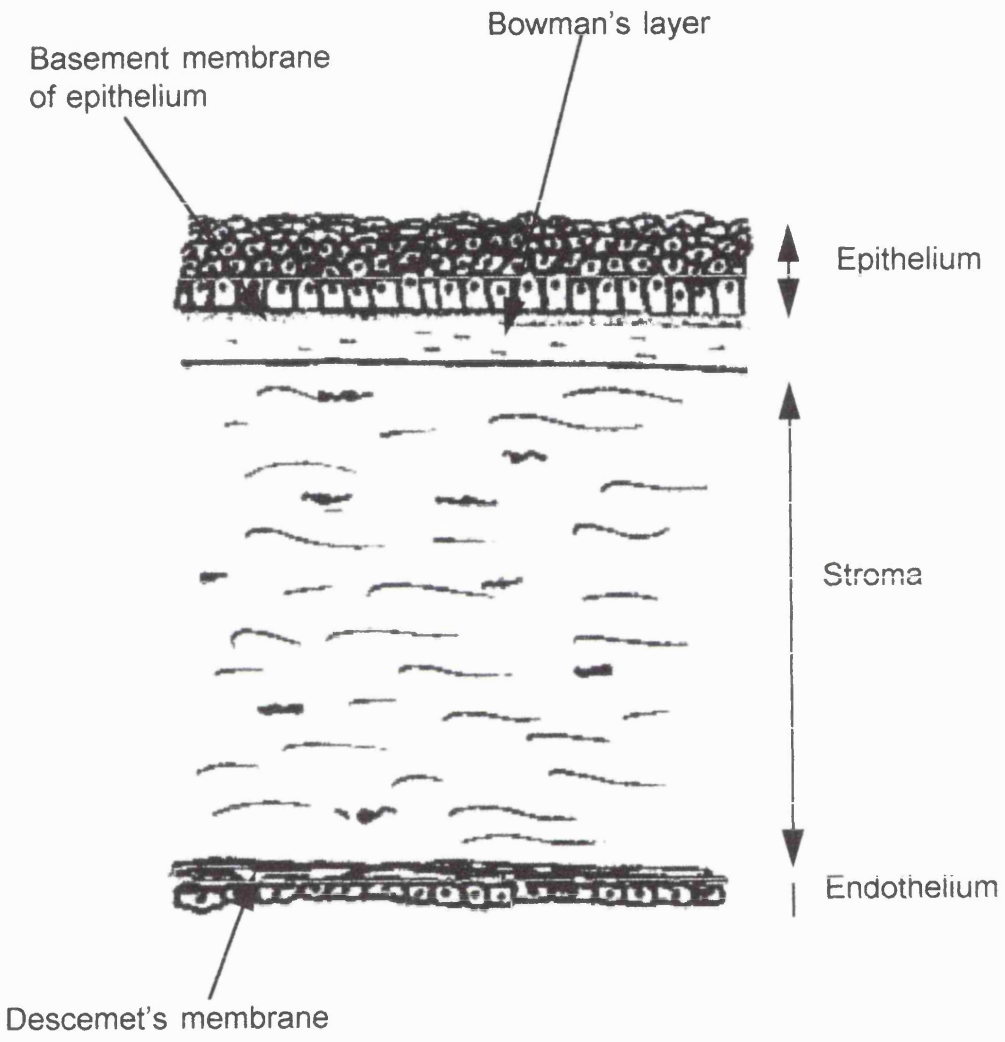


Figure 1.6. Diagrammatic representation of corneal histology

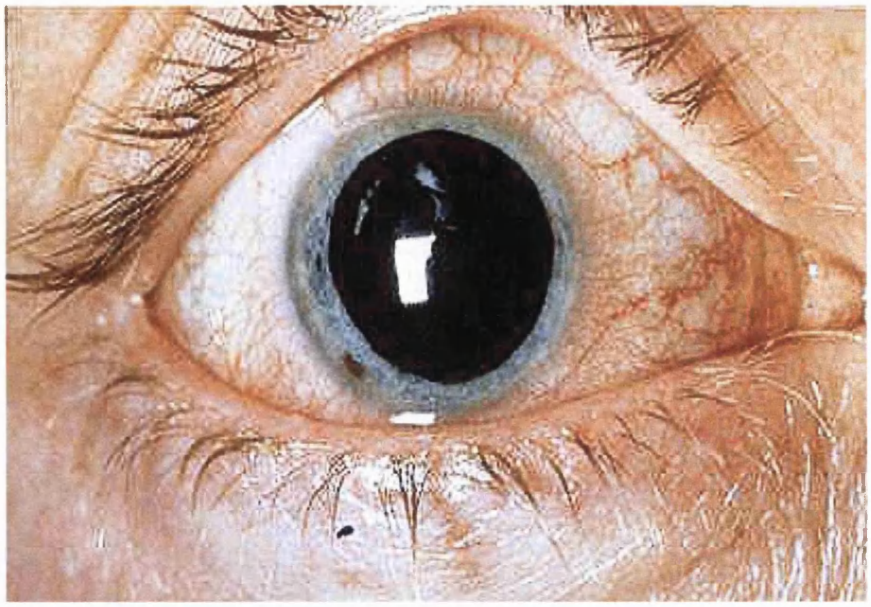


Figure 1.7a. Diffuse conjunctival hyperaemia.

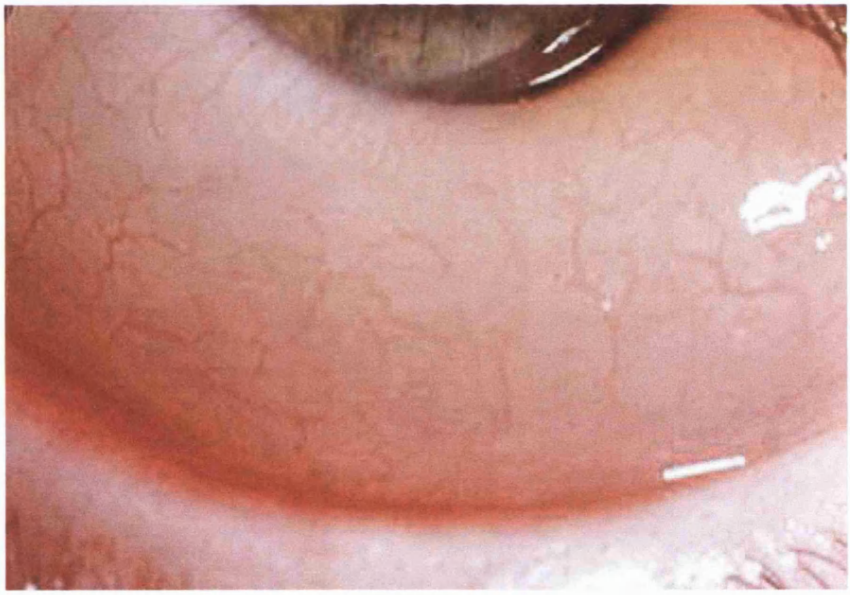


Figure 1.7b. Chemosis of the bulbar conjunctiva.

Figure 1.7. Clinical appearances in SAC.

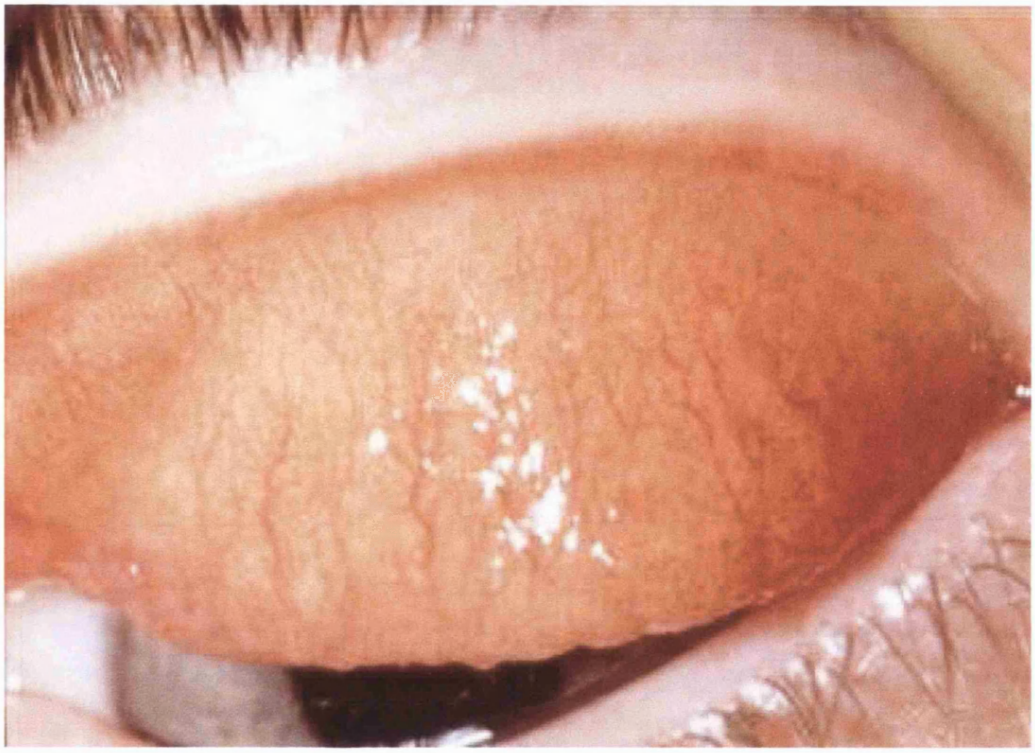


Figure 1.8. PAC: superior tarsal conjunctival hyperaemia and small papillae.

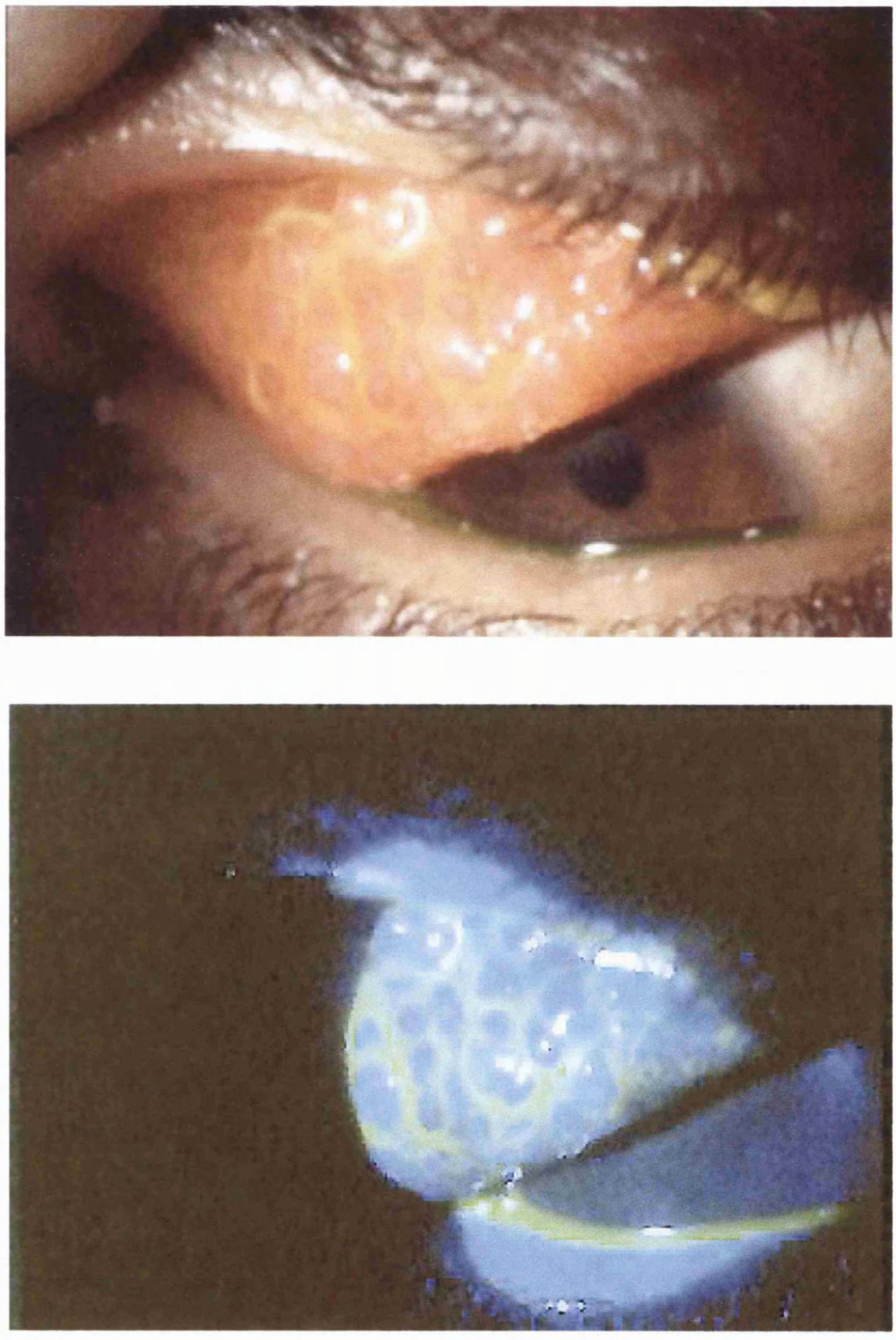


Figure 1.9. Superior tarsal giant papillae in VKC.

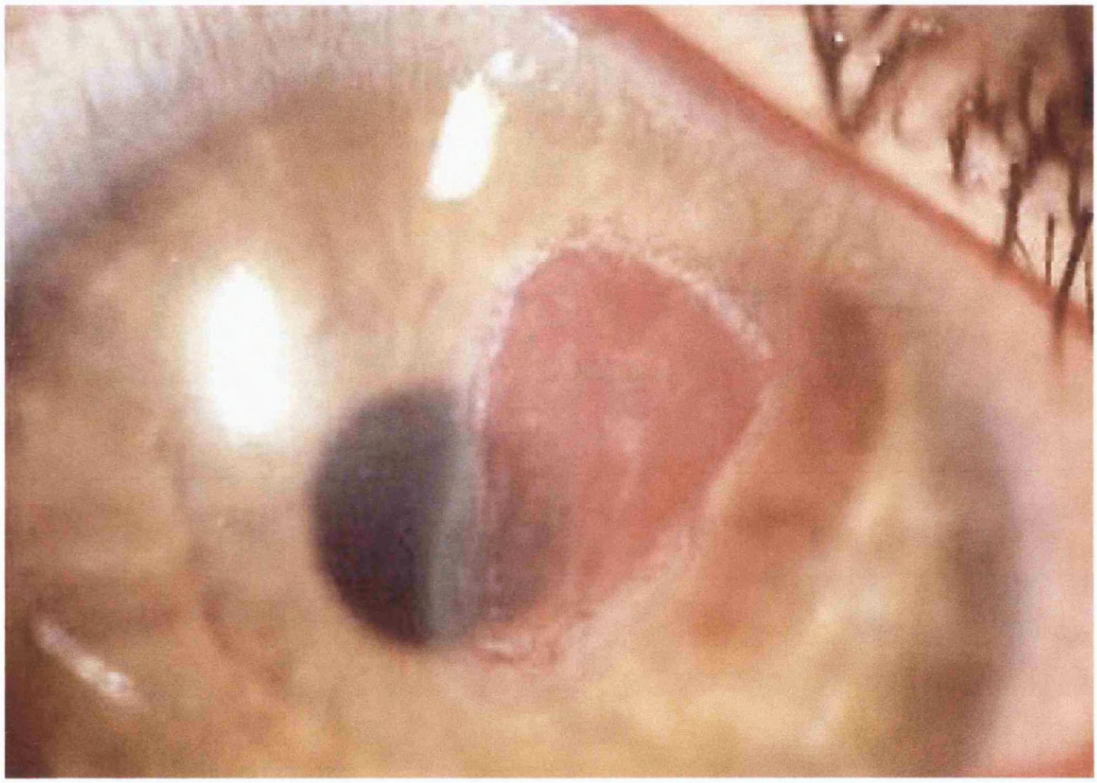


Figure 1.10. Corneal plaque ulcer stained by Rose Bengal in VKC.

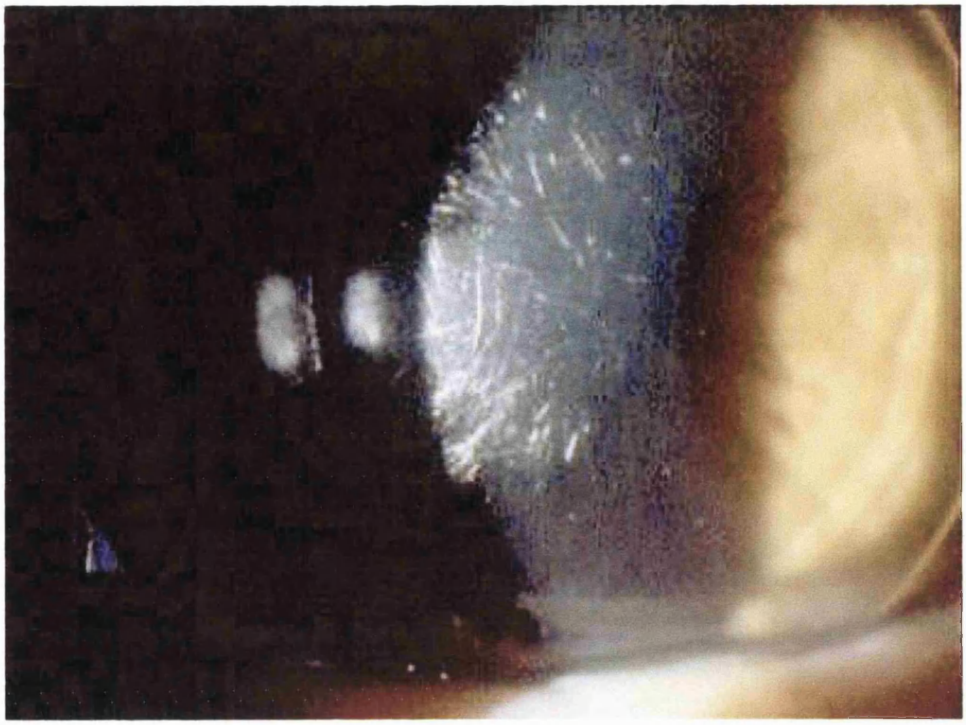


Figure 1.11. A very scratched gas permeable contact lens which caused GPC.

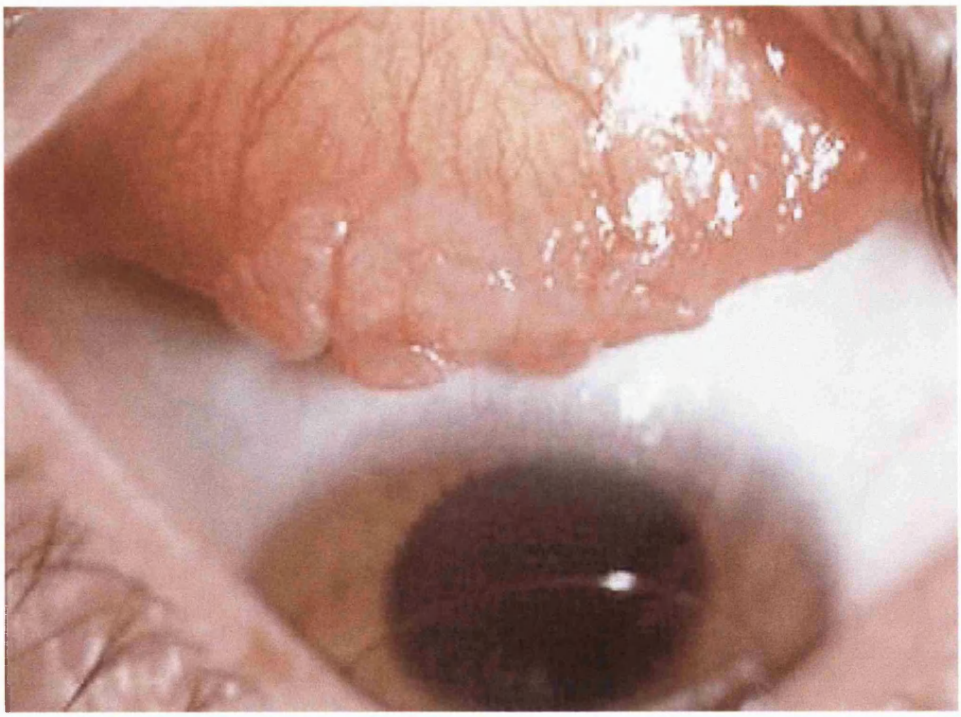


Figure 1.12. Focal GPC reaction in superior tarsal conjunctiva.

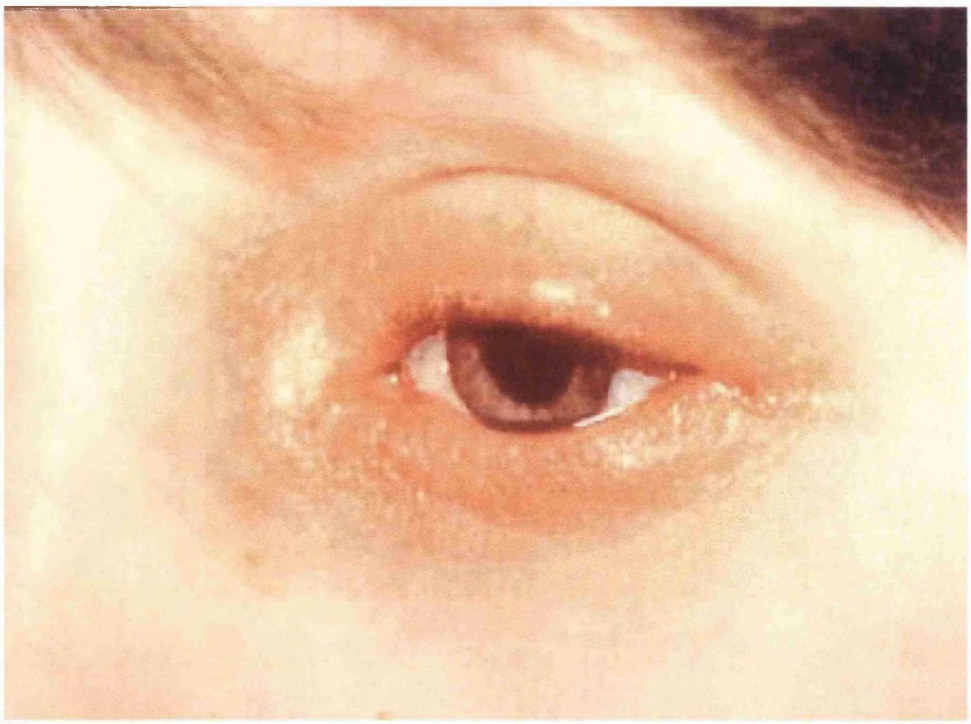


Figure 1.13. Eyelid skin dermatitis in AKC.

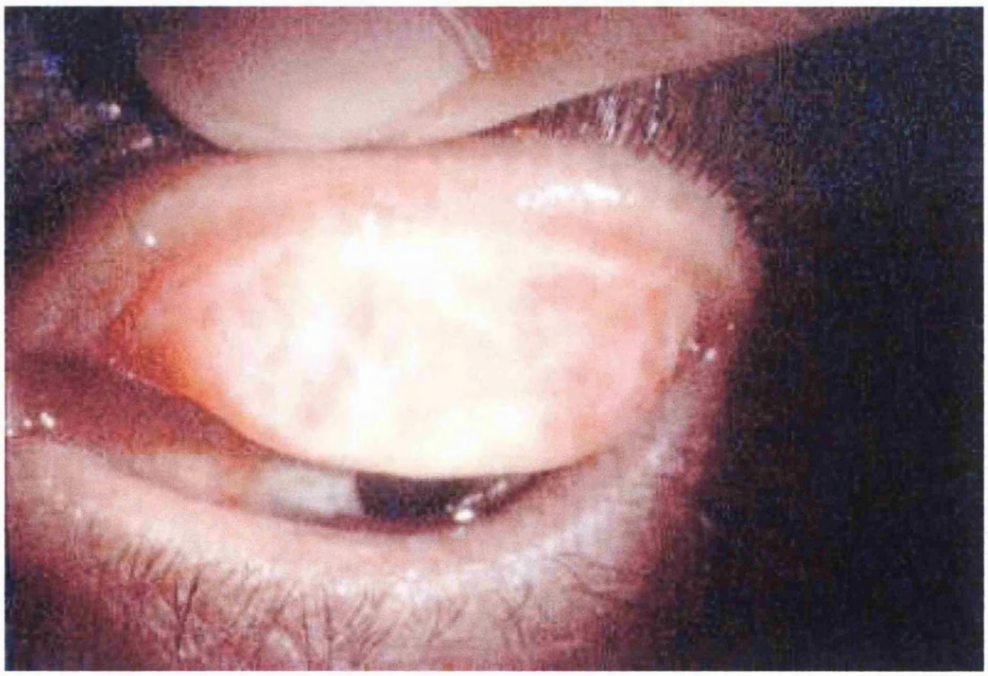


Figure 1.14. AKC superior tarsal conjunctival scarring.

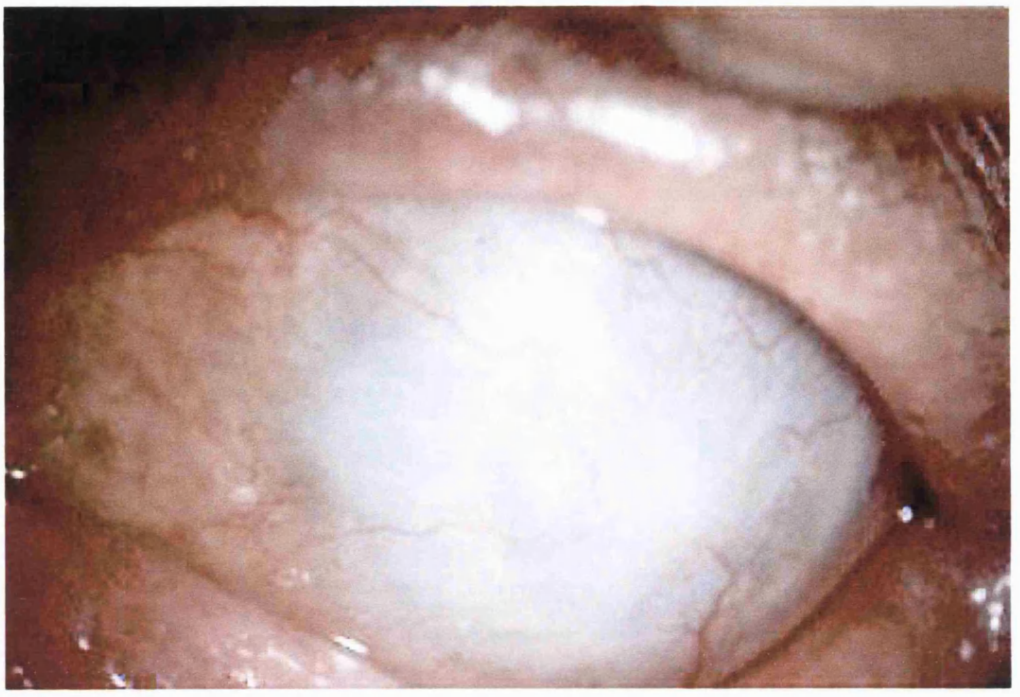


Figure 1.15. Gross AKC keratopathy.

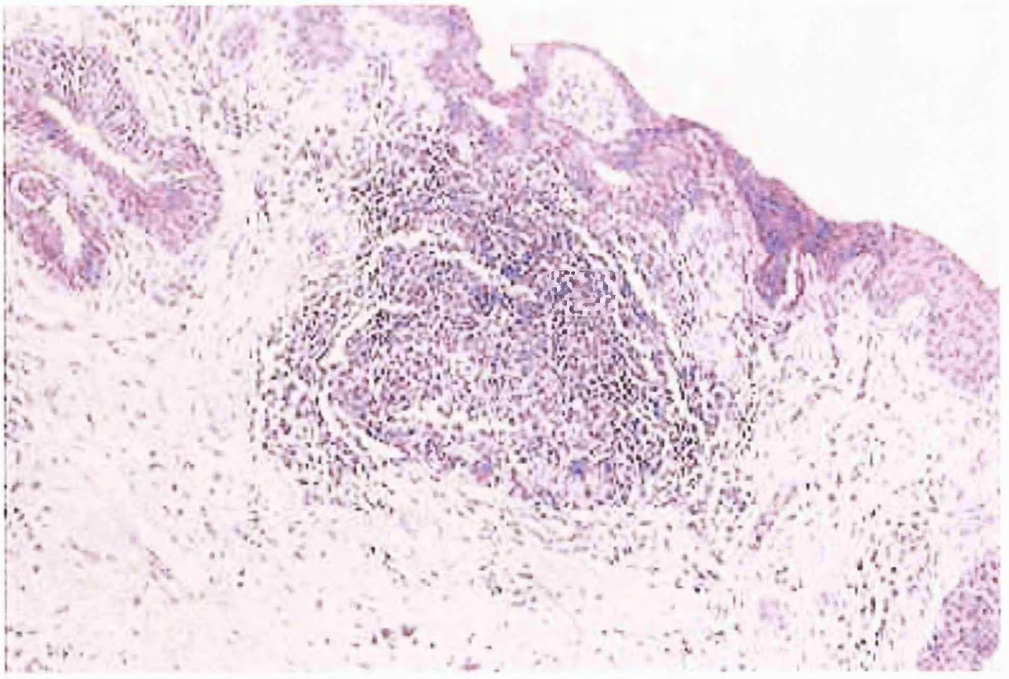


Figure 1.16. Photomicrograph demonstrating the histopathology of VKC, including a substantia propria follicle.

Table 1.1: Characteristics of rodent mast cell subtypes.

	Mucosal mast cells	**Connective tissue mast cells**
Location	mucosae lung & gut	skin, peritoneal cavity
Staining	+ after Carnoy's fixation but not formaldehyde; stain with alcian blue	+ after Carnoy's or formaldehyde; stain with alcian blue and safranin
Proteoglycan	Chondroitin sulphate di-B	Heparin
Protease	Rat mast cell protease II	Rat mast cell protease I, carboxypeptidase A
Histamine	Low	High
T cell factor (IL-3) dependent	Yes	No
Fibroblast dependent	No	Yes
C48/80 response	No	Yes
Proliferate in parasitic infection	Yes	No

Table: 1.2 Effects of mediators and cytokines upon eosinophils

Chemoattraction

Lipids	*Other*	*Cytokines*	*Chemokines*
PAF	C5a	IL-3	RANTES
LTB4	f-MLP	IL-5	MIP-1α
PGD2	histamine	GM-CSF	MCP-3, -4
HETEs		LCF	eotaxin
PF4		IL-2	IL-8

Differentiation / maturation / ↑ survival

		IL-3	
		IL-5	
		GM-CSF	

Activation

PAF	Histamine	IL-3	RANTES
LTB4		IL-5	
PGD2		GM-CSF	
PF4		IFN-γ	
		TNF-α	

↑ adherence

PAF	C5a	IL-1	
LTB4		IL-3	
PF4		IL-4	
		IL-5	
		GM-CSF	
		RANTES	
		TNF-α	

Table 1.3: Eosinophil adhesion molecules and their ligands

Eosinophil	**Endothelium**	**Matrix**
Integrins		
VLA-4 (α4β1)	VCAM-1	Fibronectin
VLA-6 (α6β1)		Laminin
α4β7	MadCAM-1	Fibronectin
CD11a (LFA-1)	ICAM-1, -2	
CD11b (Mac-1)	ICAM-1	Fibrinogen
CD11c (p150/95)	?	
Immunoglobulin-like		
PECAM	PECAM	
ICAM-3		
Selectins		
L-selectin	Gly-CAM-1, MadCAM-1, CD34	
Carbohydrate		
P-selectin ligand-1	P-selectin	
E-selectin ligand-1	E-selectin	
Others		
CD44		Hyaluronate

Table 1.4: The ocular complications of atopic dermatitis

Lids	Anterior Segment	Posterior segment
Lid dermatitis	ABC	Retinal detachment
Herpes simplex infection	AKC	
	Herpes simplex keratitis	
	Keratoconus	
	Cataracts: anterior subcapsular posterior subcapsular fleck opacities	

Garrity & Liesegang 1984, Easty et al 1975, Rich & Hanifin 1985, Foster & Colonge 1990, Donshik 1988

Table 1.5: The classification of ocular allergic diseases.

Disease	Full name	Timing	Age group	Prevalence	Keratopathy	Sight threatening	Course
SAC	Seasonal allergic conjunctivitis	Seasonal	Majority children & young adults	Very common	No	No	Mild, non-progressive, often resolves
PAC	Perennial allergic conjunctivitis	Perennial	Adult	Common	No	No	Not serious, non-progressive
VKC	Vernal keratoconjunctivitis	Seasonal - perennial if severe	Children	Uncommon	Yes	Yes	Serious, but usually resolves in 2 to 10 years with good outcome if well managed. Occasionally metamorphoses into AKC
AKC	Atopic keratoconjunctivitis	Perennial	Adults	Rare	Yes	Yes	Serious and progressive, vision often reduced
GPC	Giant papillary conjunctivitis	Related to exposure to precipitant	Adults	Uncommon	Occasionally minimal	No	Reversible with removal cause. Majority can continue with contact lens or ocular prosthesis wear with treatment

Table 1.6: Topical drugs commonly used in the treatment of ocular allergy

Drug	Tradename	Preparation	Dose	Class	Actions
Antazoline-Xylometazoline	Otrivine-Antistin	Drops	0.5%-0.05% qds	Antihistamine-vasoconstrictor combination	Competitive antagonism histamine receptor plus sympathomimetic constriction blood vessels
Antazoline-Naphazoline	Vasocon A	Drops	0.5%-0.05% qds		
Levocabastine	Livostin	Drops	0.05% bd - qds	Antihistamine	High potency H_1 receptor blockade
Sodium cromoglycate	Opticrom	Drops Ointment	2% or 4% qds 4% tds or nocte	Mast cell stabiliser	Inhibition mast cell degranulation and activity granulocytes
Lodoxamide	Alomide	Drops	0.1% qds	Mast cell stabiliser	Inhibition mast cell degranulation
Nedocromil sodium	Tilavist	Drops	2% qds	Mast cell stabiliser	Inhibition mast cell degranulation and inhibits activity granulocytes
Dexamethasone	Maxidex	Drops	0.1% alt die - every hour	Steroid	Multiple anti-inflammatory effects
Prednisolone	Predsol, PredForte	Drops	0.01-1% alt die - every hour	Steroid	Multiple antiinflammatory effects

Chapter 2

Materials and methods

2.1 Subjects

2.1.1. Consent and ethical approval

Informed consent was obtained from all participants and all procedures were approved by the Ethics Committee of Moorfields Eye Hospital.

2.1.2 Normal control subjects

Normal subjects were recruited from patients at Moorfields Eye Hospital undergoing elective squint correction or cataract surgery under general anaesthesia. Exclusion criteria were:

- a history, or current evidence, of ocular inflammatory disease or external ocular disease
- a history, or current evidence, of systemic inflammatory disorders or atopy
- the use of any medications which might affect the immunological status of the eye (e.g. anti-inflammatory or immunosuppressive drugs)
- current or previous contact lens use
- pregnancy or lactation

All subjects underwent a full ophthalmic examination to ensure the ocular surface had a normal clinical appearance.

For the initial study of normal conjunctiva, 20 normal adult patients (12 female, mean age ±SEM [age range] 59.3 ±4.0 [18-87] years) were recruited (Appendix 1). For the studies comparing normal tissue findings with those in ocular allergic disease, 20 normal, non-age- or sex-matched subjects (11 female, age 38.1 ±5.6 [5-85] years) were recruited.

2.1.3 Subjects with ocular allergic disease

Patients with ocular allergic disease were recruited prospectively from the External Disease Service of Moorfields Eye Hospital. Exclusion criteria were:

- a history, or current evidence, of unrelated ocular inflammatory disease or external ocular disease
- a history, or current evidence, of non-atopic systemic inflammatory or immunological disorders
- the use of any systemic medications (e.g. anti-inflammatory or immunosuppressive drugs) which might affect the immunological status of the eye or its response to anti-allergic treatment
- pregnancy or lactation

Conjunctival biopsies were obtained from subjects with VKC (n=18, 5 female, age 19.4 ±1.0 [12-29] years), GPC (n=18, 10 female, 38.9 ±4.2 [20-73] years) and AKC (n=18, 7 female, age 29.5 ±1.9 [21-46] years) (Appendix 2). All had active disease. Patients with GPC were biopsied before the onset of treatment, and patients with VKC and AKC had received no topical steroids for 3 months and no topical cromones for one week prior to biopsy. The diagnoses were made clinically (Ben Ezra et al., 1994; Buckley, 1981; Foster and Calonge, 1990; Ehlers and Donshik, 1992; Buckley, 1988b). All patients with AKC also suffered from atopic dermatitis and all patients with VKC or AKC had current or previous corneal involvement.

The age in each group was not significantly different from that in the controls, but the age in VKC was statistically lower than in GPC ($p<0.001$) and AKC ($p<0.001$) as might be expected given that VKC is a disease of childhood (Buckley, 1989). The age of VKC patients was higher than might be expected (Buckley, 1989; Ben Ezra et al., 1994) as ethical approval of this study demanded patients to be age 12 years or over (to ensure co-operation with tissue harvesting under local anaesthetic). The increased number of males in the VKC group is expected (Buckley, 1989) but did not reach statistical significance compared with controls or the other disease groups.

The duration of the disease was 4.7 ±0.9 years in GPC, which was significantly shorter than for VKC (8.6 ±1.2 years, $p=0.009$) and AKC (16.4 ±2.5 years, $p=0.0001$). The duration of disease in VKC was significantly less than for AKC ($p=0.009$).

2.1.4 Subjects with AKC in trial of CsA

2.1.4.1 Clinical trial of CsA in steroid-dependent AKC

Twenty-one patients with steroid-dependent AKC (9 female, age 34.7 ±2.9 [18-66] years) were recruited prospectively from the External Disease Service of Moorfields Eye Hospital. All the patients had been unable to cease the use of topical steroids for the treatment of AKC, despite the use of other agents (e.g. mast cell stabilisers, lubricants, treatment for blepharitis and lid skin eczema) designed to minimise the steroid requirement (Hingorani and Lightman, 1995).

Exclusion criteria were

- a history, or current evidence, of non-atopic systemic inflammatory or immunological disorders
- the use of any systemic medications (e.g. anti-inflammatory, steroid or immunosuppressive drugs) which might affect the immunological status of the eye or its response to anti-allergic treatment

- the use of steroid eyedrops for reasons other than allergic eye disease (e.g. post-intraocular surgery)
- patients who had undergone ocular surgery within the previous 6 months
- pregnancy or lactation

Of the 21 patients in the trial, 12 were assigned to receive CsA and 9 to receive placebo. In the CsA group there were 4 females and the mean age (±SEM) was 33.9 (±3.9) years. In the placebo group there were 5 females and the age was 35.7 (±4.6) years. There were no significant differences between the ages and sexes of the two groups.

2.1.4.2 Immunomodulatory effects of CsA in AKC

Eight patients (2 female, age 33.2±4.8 [22-61] years) with active AKC were prospectively recruited. Exclusion criteria were as above (section 2.1.4.1).

2.2 Biopsies

All subjects with ocular allergic disease had active disease and none had received topical steroids for 3 months or cromones for 1 week prior to biopsy. In the affected patients, anaesthesia of the superior tarsal conjunctiva was achieved with topical amethocaine 1% and local infiltration with 2% lignocaine and adrenaline. In the controls, the biopsy was harvested just before the surgical procedure began (under general anaesthetic). A 3mm trephine was used to obtain a sample from the central third of the superior tarsal conjunctiva (Fig. 2.1), which is the site of maximum inflammatory conjunctival involvement in these disorders. The tarsal biopsy was immediately divided into two or three portions, to allow analysis of the same tissue by different methods (tissue staining, immunohistochemistry [IHC], *in situ* hybridisation [ISH] and cell culture [data not presented here]). Bulbar specimens $3mm^2$ in size were taken with scissors from the superotemporal bulbar conjunctiva in a proportion of normal controls.

For tissue staining and IHC, fresh tissue was immediately snap-frozen in Tissue-Tek OCT (BDH) and stored at -70°. Sections of biopsy for ISH were immediately snap frozen and stored at -70°C. Other sections of biopsy for tissue staining and IHC were placed immediately in ice-cooled acetone containing iodoacetamide (20mM) and phenylmethylsulfonylfluoride (2mM) protease inhibitors, stored overnight at -20°C, then processed for, and embedded in, glycol methacrylate (GMA) resin (TAAB Laboratories, Aldermaston, UK) (Britten et al., 1993). The blocks were stored at -20° C.

2.3 Immunohistochemistry

2.3.1 Immunohistochemistry on frozen sections: one-colour IHC

6μm cryostat sections were cut, which were placed on amino-propyl triethoxy silane (APES, Sigma Chemicals, Poole, U.K.) coated slides and allowed to air dry for 1 to 3 hours. An avidin-biotin-peroxidase complex technique was used and all reagents were contained in a Vectastain ABC-peroxidase staining kit (Vector Laboratories, Peterborough, UK) unless otherwise stated. After fixation in acetone for 10 minutes, the slides were washed in PBS (phosphate buffered saline, Sigma Chemicals, Poole, UK) and incubated with 1:10 normal horse serum (Dako Ltd., High Wycombe, U.K.) for 20 minutes to block non-specific staining. The sections were then incubated with one of a panel of primary mouse anti-human antibodies (IgG1) at an appropriate dilution (Appendix 4) for 30 minutes. After washing with PBS, a secondary biotinylated horse anti-mouse antibody was applied to the sections for 20 minutes. Following a further wash in PBS, the slides were incubated with a preformed avidin-biotin-horse radish peroxidase macromolecular complex for 45 minutes and the reaction product was developed with amino-ethyl carbazole (Sigma) and peroxide. The slides were rinsed in water, counterstained with Mayer's haematoxylin and mounted with glycergel (Dako). The same procedure was performed on tonsil sections (obtained previously) with and without primary antibodies against CD4 for positive and negative controls respectively.

Haematoxylin and eosin (H&E) staining (Fig. 2.2) was employed for morphological definition and orientation of the sections and to identify plasma cells.

2.3.2 Immunohistochemistry on resin sections: one- and two-colour IHC

An ultramicrotome was used to cut 2μm sections which were floated on 0.2% ammonia water, placed on APES coated slides and allowed to air dry. An indirect streptavidin-biotin-peroxidase complex technique was used. Endogenous peroxidase was inhibited using a solution of 0.3% hydrogen peroxide in 0.1% sodium azide (Sigma) for 10 minutes followed by 2 five-minute washes in PBS and incubation for 30 minutes with 10% normal rabbit serum (Dako) to prevent non-specific binding of monoclonal antibody. The sections were then incubated overnight with one of a panel of mouse anti-human antibodies (IgG1) at appropriate dilutions.

After washing in PBS, biotinylated anti-mouse antibody (Dako) was applied to the sections for 2 hours. Following a further wash in PBS, the slides were incubated with streptavidin-peroxidase (Dako) for 2 hours, again washed in PBS and developed for 25

minutes with a solution of amino-ethyl carbazole (AEC, Sigma) and peroxide. For cell-counts (one-colour IHC), the slides were then rinsed in tap water, counterstained with Mayer's haematoxylin and mounted with Glycergel (Dako). For the localisation of antigens to leukocytes (two-colour IHC), the slides were washed with PBS and the immunohistochemistry process was repeated using mouse anti-human antibody (against eosinophil cationic protein or against CD3) as the primary antibody for identification of eosinophils or T cells, biotinylated anti-mouse antibody (Dako) as the secondary antibody and diaminobenzamine (DAB, Vector) instead of AEC as chromagen. Once again, incubation with the primary antibody was preceded by exposure of sections to non-specific rabbit serum to minimise non-specific binding. Immunohistochemistry was performed on tonsil sections with and without primary antibodies against CD4 for positive and negative controls respectively. Immunohistochemistry was performed on conjunctival sections with an unrelated mouse monoclonal antibody of the same isotype (anti-cytomegalovirus, Dako) and substituting normal rabbit serum for primary antibody for negative controls, and performed using an anti-cytokeratin monoclonal antibody (epithelial marker) as the primary antibody for a positive control.

Haematoxylin and eosin (H&E) staining was employed for morphological definition and orientation of the sections and to identify plasma cells.

2.3.3 Cell counting

After one-colour IHC, stained cells were identified by a red deposition of AEC reaction product (Figure 2.3). After two-colour IHC, eosinophils or T cells were identified by the black-staining DAB reaction product, and eosinophils or T cells which stained for cytokines or cell surface antigens by a brown or combined red-black colour due to the combination of a positive reaction to AEC and DAB (Figure 2.4). Plasma cells were identified by their characteristic morphology on H&E sections (large amounts of agranular cytoplasm with a pale staining perinuclear area, peripherally-clumped chromatin in an eccentric nucleus).

Grading of the degree of staining and cell counts were performed in a masked fashion with an Olympus BH2 microscope using a $1mm^2$ eyepiece graticule at x400 magnification. For each section, cells were counted in at least 3 adjacent fields (as limited by size of specimen, preferably 8 to 10 fields when possible) and expressed as mean counts per mm^2 of substantia propria or per mm of epithelium. For two-colour IHC, in the majority of studies the results were expressed as the number of samples containing any double-staining cells and as the percentage of eosinophils or T cells in each sample which were double-stained, so that comparisons could be made between

the disorders, where there were different degrees of T cell or eosinophil infiltration. In the one study where samples from the same disorder (AKC, Chapter 8) were compared, the results were expressed as the numbers per square mm of double-stained cells, to allow analysis of results from a small number of samples with similar T cell densities.

2.3.4 Grading of epithelial cell staining

Epithelial cells staining positively were identified by a red deposition of AEC reaction product (Fig. 2.5). Grading of the degree of staining was performed by one masked observer with an Olympus BH2 microscope at x400 magnification on 3 or more adjacent fields (limited by the size of the sections). As the epithelial cell layer thickness (number of epithelial cell layers) is variably increased in chronic allergic eye disease (Morgan, 1971; Allansmith et al., 1981b), it was deemed not accurate to simply count the numbers of positively stained epithelial cells per high powered microscopic field. For each section, positive immunostaining of epithelial cells was graded using a four-point scale: 0 (absent), 1 (positivity on ≤ 25% of epithelial cells), 2 (positivity on 25 to 50% cells), 3 (positivity on 50 to 70% cells), 4 (positivity on 75 to 100% epithelial cells) (Ciprandi et al., 1993a). Results were expressed as the number of samples containing positive staining epithelial cells and as the mean staining grade.

2.4 *In situ* hybridisation

All the in situ hybridisation was performed by Daniella Metz.

cDNA for IL-2, -3, -4 and -5 in pGEM vectors (kind gift from Prof. A.B. Kay, National Heart and Lung Institute, London, U.K.) were linearised with Hind III, EcoR I, Bam H I, Xba I and Sph I and these templates were used to synthesise ^{35}S-labelled sense and anti-sense riboprobes using T-7 and SP-6 RNA polymerases.

All *in situ* reagents were from Sigma Chemicals unless otherwise stated. Cryostat sections (10μm) were mounted on APES coated RNase-free slides and fixed in freshly prepared 4% paraformaldehyde in PBS for 20 minutes at room temperature. After two washes in 15% PBS-buffered sucrose, the tissue was permeabilised with 0.3% Triton X-100 in PBS for 10 minutes and then proteinase K (1μg/ml in 0.1M Tris, pH 8.0) for 10 minutes. The reaction was stopped with 4% paraformaldehyde in PBS. After washing in PBS, sections were treated with 10mM iodoacetamide and 10mM N-ethylmaleimide for 30 minutes at 37°C followed by 0.25% acetic anhydride in 0.1M triethanolamine for 15 minutes to reduce non-specific binding. Prehybridisation was carried out with 50% formamide in 2X standard saline citrate (SSC) for 30 minutes at 37°C. A 20μl hybridisation mixture containing 0.5-1 x 10^6cpm (~0.5ng RNA/ml)

radiolabelled probe, 100mM dithiothreitol and hybridisation buffer (50% deionised formamide, 5X SSC, 10% dextran sulfate, 5X Denhardt's solution, 0.5% sodium dodecylsulfate & 100μg/ml sheared denatured salmon sperm) was added to each slide. The sections were covered with silica (Repelcote, BDH, Merck Ltd., Poole, U.K.)-coated cover slips and hybridised at 42°C in a humid chamber overnight. After hybridisation, slides were washed in 4X SSC for 20 minutes followed by incubation with 4X SSC containing 10μg/ml RNase-A for 30 minutes to remove any non-hybridised single-stranded RNA. The slides were subsequently washed in decreasing concentrations of SSC (4X to 0.1X SSC) at 40°C.

The slides were dehydrated with graded ethanol solutions containing 0.3M ammonium acetate, then dipped in photographic emulsion (K5, Ilford, U.K.) and exposed for 12 days at 4°C. The slides were developed in Phenisol developer (Ilford) and fixed in IN-1 fixer (Ilford), then counterstained in Mayer's haematoxylin and viewed by light microscopy (Fig. 2.7).

Cytospins were prepared from a T cell clone (2H22, kind gift from D.J. Quint, Glaxo-Wellcome Laboratories, Stevenage, U.K.) from a patient with hyper-IgE syndrome, known to produce IL-2, -3, -4 and -5 upon concanavalin A (5μg/ml) stimulation, and were used as positive controls (Fig. 2.7). Hybridisations with sense probes or with anti-sense probes after pretreatment with RNase-A were used for negative controls. Positive cells were not seen in the negative controls (Fig. 2.8).

Accurate counting of positive cells was not possible due to the thickness of the sections (10μm). Therefore biopsies where there were clearly more than 3 positive cells (an arbitrary cut-off determined from our previous experience with ISH in conjunctiva) were rated qualitatively as positive and the number of positive biopsies in each disease group determined for comparison.

2.5 Clinical trial of topical CsA in AKC

Details of these methods are give in chapter 7 and patient details in Appendix 3.

2.6 Statistical analysis

Non-parametric statistical tests (Mann-Whitney U test, two-tailed or Wilcoxon signed rank test) were employed to analyse the differences in cell numbers and percentages and differences between staining grades between the subject groups, and Fisher's exact test (two-tailed) to analyse differences in the numbers of samples, a p value of <0.05

being taken as statistically significant. Data from the clinical trial of CsA in AKC was analysed using Mann-Whitney U test and the Fisher's exact test (two-tailed).

Chapter 2: Figures

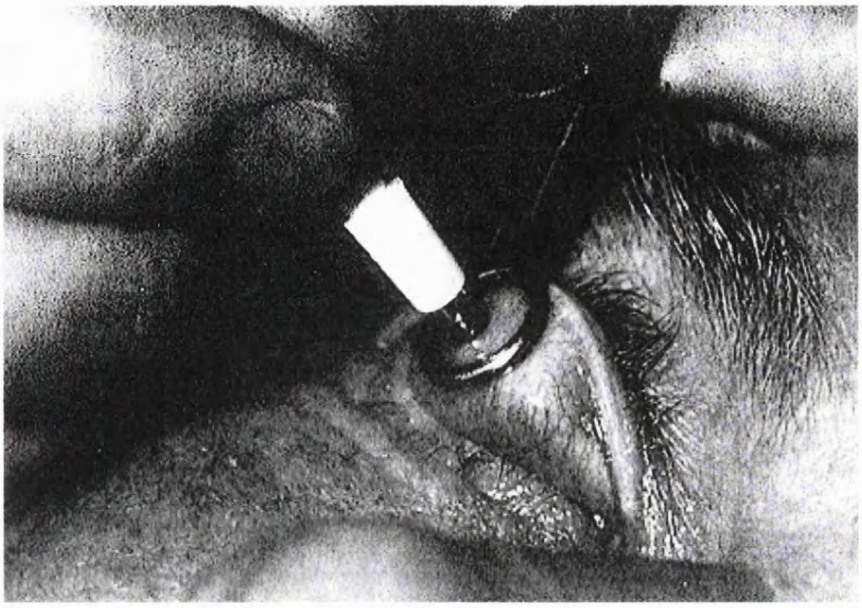


Figure 2.1a. Biopsy of superior tarsal conjunctiva.

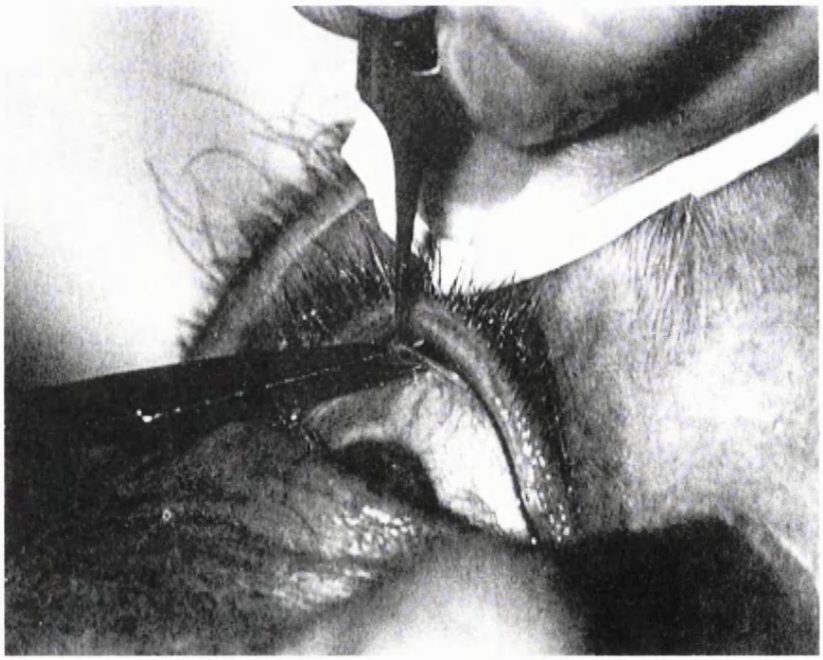
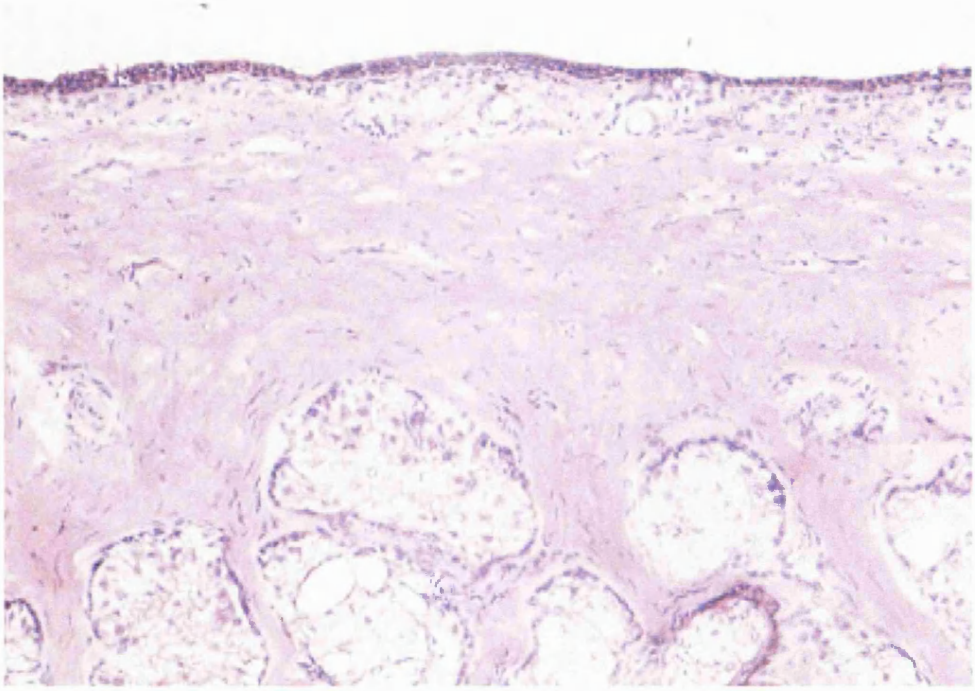
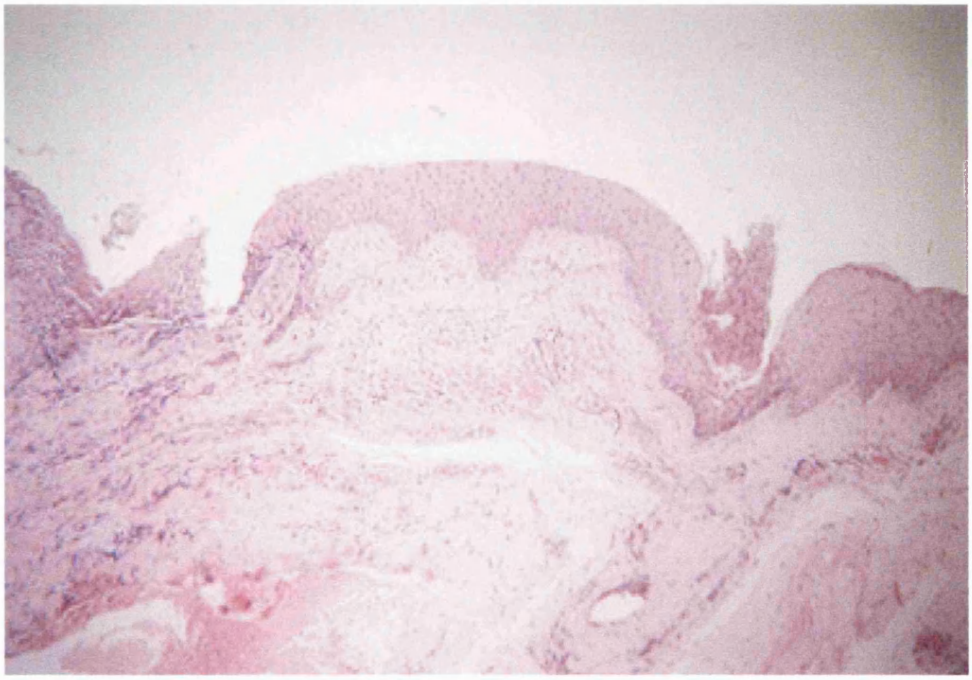


Figure 2.1b. Biopsy of superior bulbar conjunctiva.

Figure 2.1. The method of obtaining conjunctival biopsy specimens.

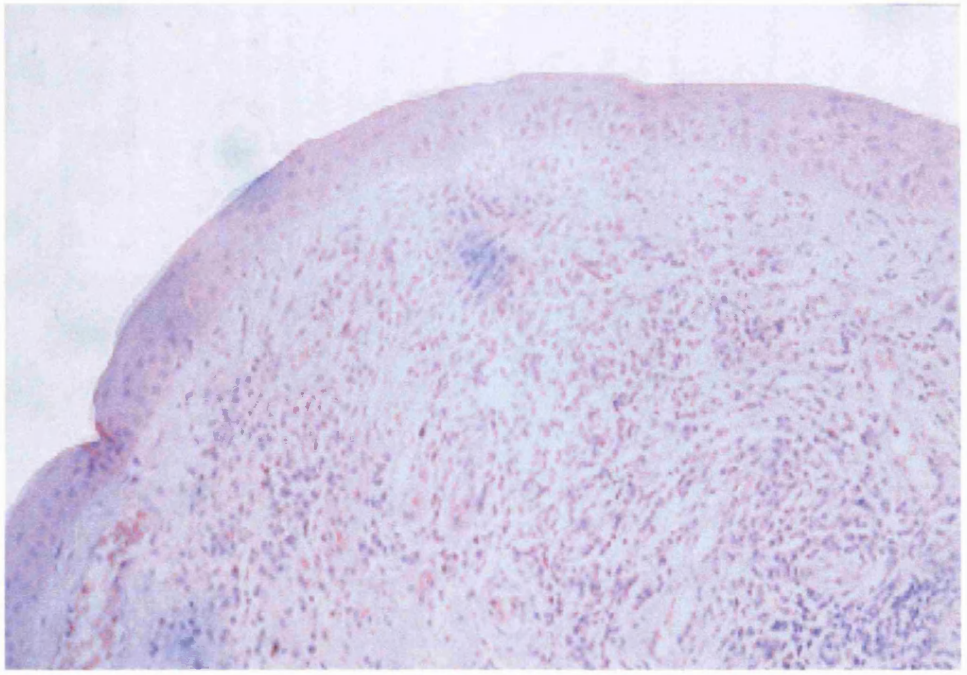


Normal

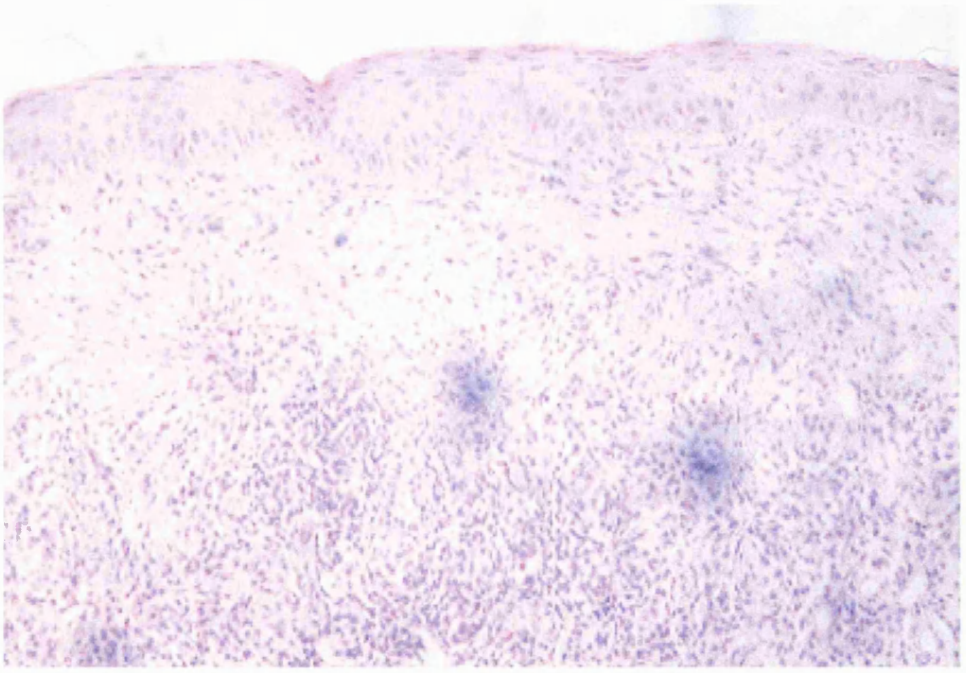


VKC

Figure 2.2. H & E staining of frozen sections from normal, VKC, GPC and AKC tarsal conjunctival specimens (x40).



GPC



AKC

Figure 2.2 (cont.).

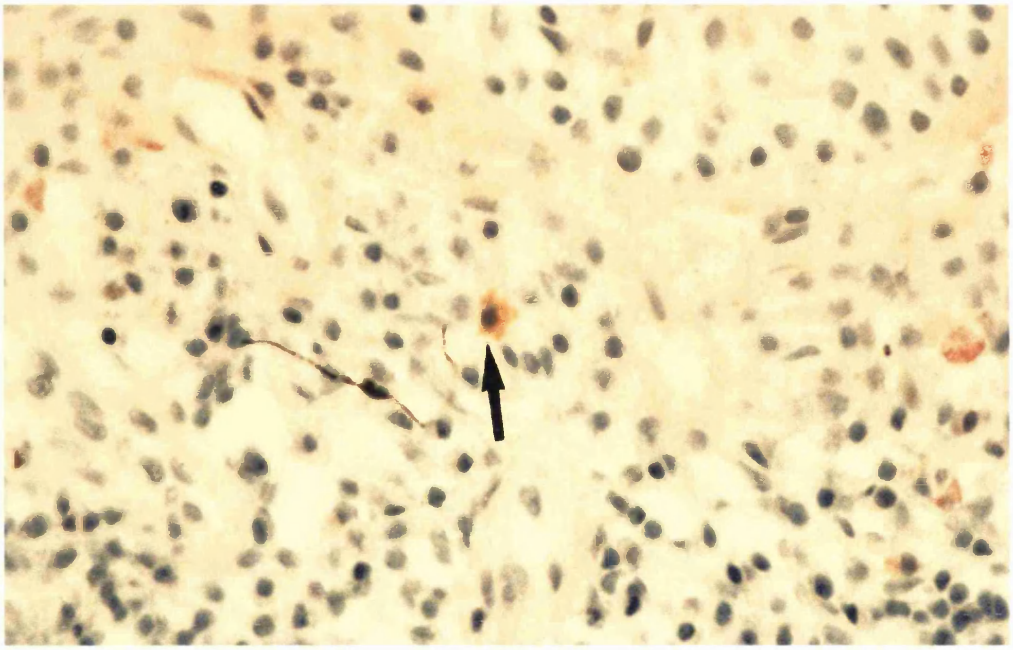


Figure 2.3. One-colour IHC demonstrating red AEC reaction product ($CD4^+$) (x200).

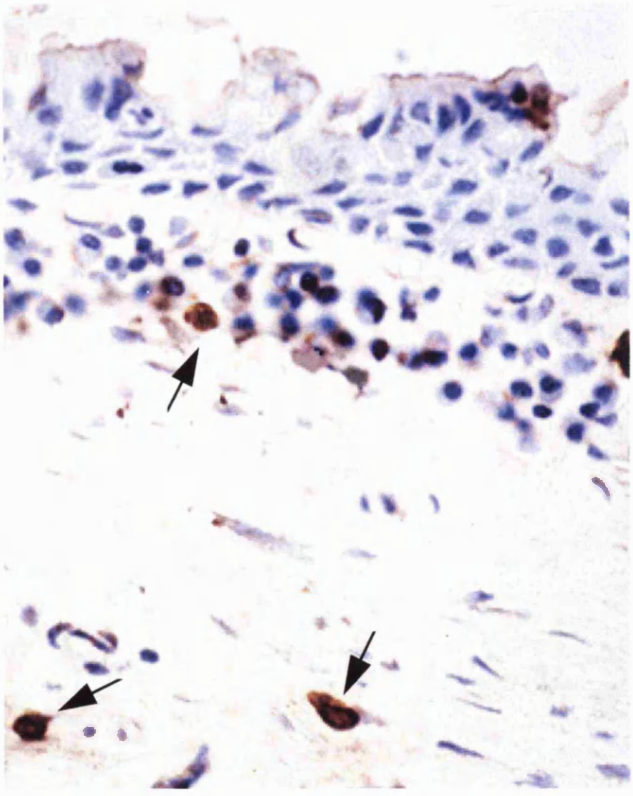


Figure 2.4. Two-colour IHC demonstrating combined AEC (red, $IL\text{-}3^+$) and DAB (black, $CD4^+$) reaction products (x200).

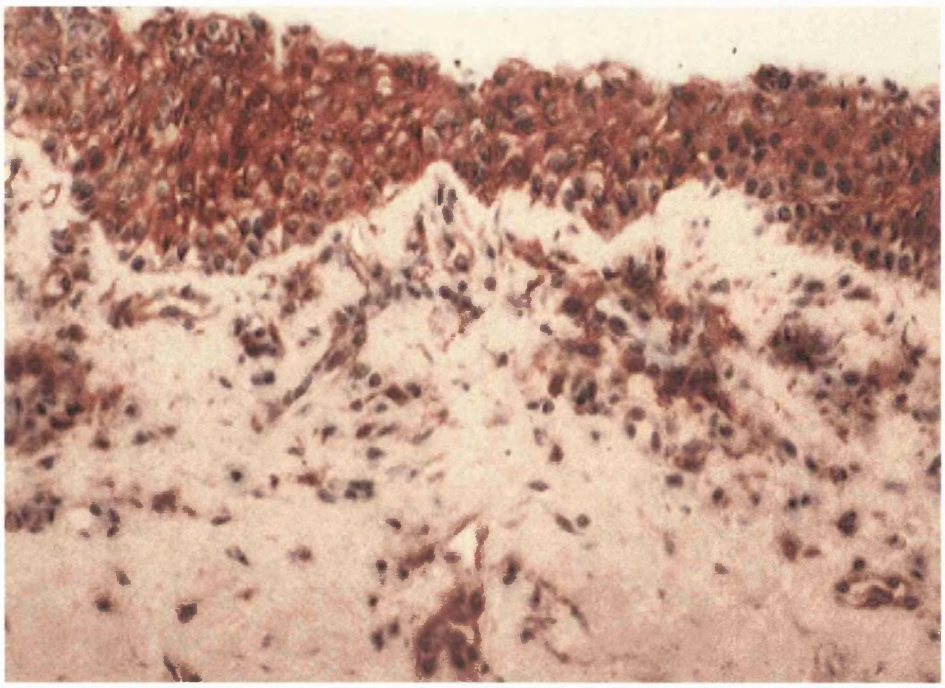


Figure 2.5. Red AEC reaction product deposition on conjunctival epithelial cells staining positive for RANTES (x200).

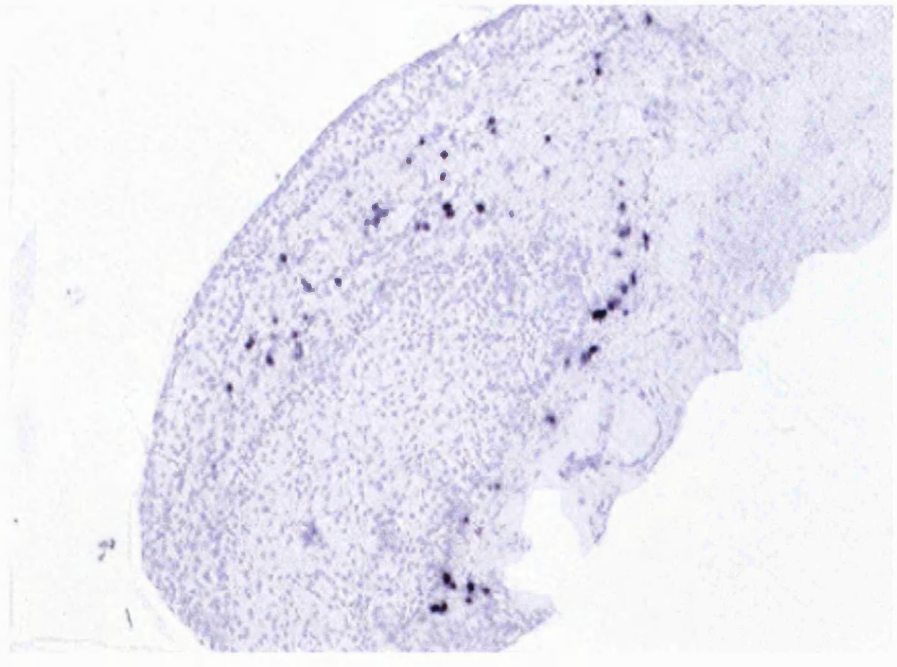


Figure 2.6. In situ hybridisation demonstrates foci of IL-4 mRNA localisation (x40).

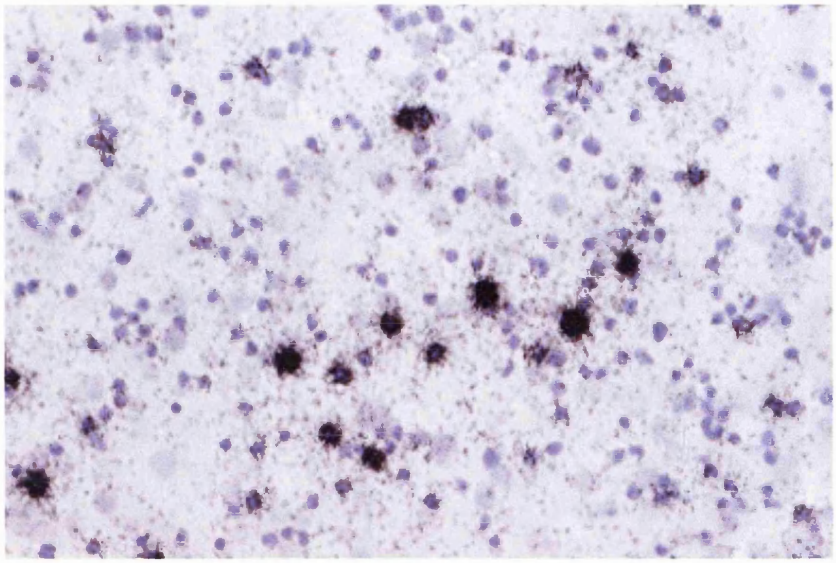


Figure 2.7. Foci of IL-4 mRNA localisation in positive control T cell cytospin (x100).

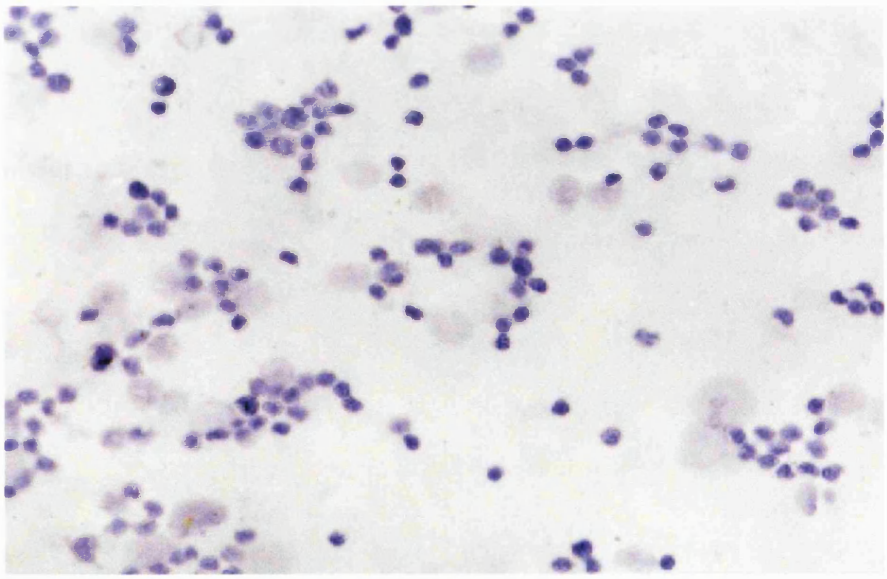


Figure 2.8. No IL-4 mRNA localisation in negative control T cell cytospin (x100).

Chapter 3

Characterisation of the normal conjunctival leukocyte population

3.1 Introduction

3.1.1 Leukocytes in the conjunctiva and ocular defence

The conjunctiva is in contact with the external environment and therefore constantly exposed to many antigens and allergens (Chandler, 1996). Non-specific defence of the ocular surface is provided by the bony orbit, lids and cilia, the aqueous tears and tear film mucus, commensal micro-organisms and the presence of natural killer cells, neutrophils and macrophages (Chandler and Gillette, 1983; Smolin, 1985). Antigen-specific defence relies on conjunctival lymphoid cells and mast cells. (Roitt et al., 1993; Watt and Ward, 1985) Previous studies have shown a large leukocyte population in the normal conjunctiva and suggested the presence of organised CALT (Allansmith et al., 1978; Dua et al., 1994; Wotherspoon et al., 1994).

3.1.2 Objectives

An understanding of the structure and cellular composition of non-pathological conjunctival lymphoid tissue and other leukocytes is a prerequisite for the future study of inflammatory ocular surface disorders. In this study, immunohistochemical techniques were used to localise and identify leukocytes present in the tarsal and bulbar conjunctiva, with a particular emphasis on the lymphocyte population.

3.2 Methods

This work was performed in conjunction with Daniella Metz.

3.2.1 Subjects

Conjunctival biopsies were obtained from 20 normal, non-atopic adult patients (12 female, age 59.3 ±4.0 [18-87] years) undergoing routine ocular surgery. The subjects had no ocular or systemic inflammatory disease, no external ocular disease and had never worn contact lenses. Tarsal biopsies were collected from 12 patients (age 63.2 ± 5.1 years) and paired tarsal and bulbar biopsies from 8 patients (age 53.2 ±6.4 years).

3.2.2 Biopsies

Superior tarsal conjunctival specimens (3mm diameter) and superotemporal bulbar conjunctival specimens ($3mm^2$) were harvested prior to the commencement of surgery. The fresh tissue was immediately snap-frozen in Tissue-Tek OCT (BDH) and stored at -70°C.

3.2.3 Immunohistochemistry

6μm cryostat sections were used for one-colour IHC. A panel of primary mouse anti-human antibodies (table 3.1) were used. Haematoxylin and eosin (H&E) staining was

employed for morphological definition and orientation of the sections and to identify plasma cells.

3.2.4 Cell counts and statistical analysis

After IHC, positively staining cells were identified by morphology and a red deposition on the cell surface. Plasma cells were identified by their characteristic morphology on H&E. Cell counts were performed by a masked observer and were expressed as mean counts per mm of epithelium or per square mm of substantia propria. A non-parametric statistical test (Wilcoxon signed rank test) was used to analyse the differences in mean cell counts.

3.3 Results

3.3.1 General

There was a great variation in the number of leukocytes present in the normal conjunctiva. The majority of mononuclear cells were situated in the substantia propria directly under the epithelium. One bulbar specimen demonstrated a single lymphoid aggregate directly under slightly flattened epithelium lacking goblet cells (Figure 1). There were no demonstrable effects of age or sex on the cell numbers and distribution.

3.3.2 Tarsal biopsies (n=20)

Cells staining positive for CD3 were present in the substantia propria and epithelium (mainly in the basal layers) of all tarsal specimens (figure 2) and were the predominant cell type (Table 3.2). CD45Ro expression was significantly lower than CD3 expression in the substantia propria (CD45Ro/CD3 = 0.57) whereas in the epithelium $CD45Ro^+$ and $CD3^+$ cell numbers were not significantly different (CD45Ro/CD3 = 0.75).

$CD4^+$ and $CD8^+$ cells were present in the stroma and the epithelium of normal tarsal conjunctiva. There were similar numbers of $CD4^+$ and $CD8^+$ cells in the substantia propria (CD4/CD8 = 1.3) but $CD8^+$ cells were significantly more frequent than $CD4^+$ in the epithelium (CD4/CD8 = 0.3). Cells with positive staining for IL-2R occurred infrequently in the stroma, with only 4 specimens containing such cells, and none were observed in the epithelium. No TCRγδ or HML-1 positive cells were seen in the epithelium but 2.2% of substantia propria $CD3^+$ cells were positive for TCRγδ and 12% of $CD8^+$ cells bore HML-1.

Few specimens revealed staining for scattered $CD20^+$ B cells; plasma cells and mast cells were also present in small numbers and all these cell types were confined to the

substantia propria. In 12 of the 20 tarsal biopsies, NK cells were seen in the substantia propria.

Cells expressing the macrophage marker CD68 were also detected throughout the conjunctival layers and were the second most frequently occurring cell type. MHC class II antigens were present on a large number of mononuclear cells, their numbers and distribution correlating well with those of $CD68^+$ cells. In addition, large dendritic cells in the epithelium and occasional epithelial cells stained for HLA-DR. Staining for ECP was negative in all tissues, but neutrophils were regularly present in the substantia propria.

3.3.3 Tarsal versus bulbar cell counts (n=8)

Greater numbers of leukocytes were found in bulbar epithelium and substantia propria than in the corresponding tarsal areas (Table 3.3). As in the tarsal biopsies, the most frequently occurring cell was the $CD3^+$ T cell, followed by macrophages, and the distribution of CD4 and CD8 was also similar, with $CD8^+$ cells outnumbering $CD4^+$ cells in the epithelium (CD4/CD8 = 0.3) but not the substantia propria (CD4/CD8 = 2). Numbers of B cells, plasma cells and granulocytes were similar in tarsal and bulbar specimens.

The expression of CD3 (figure 3), CD25 and CD57 (NK cells) was significantly greater in the substantia propria of bulbar conjunctiva. The staining for CD45Ro was also increased, although this did not reach statistical significance. The relationship of CD45Ro staining with the numbers of $CD3^+$ cells was similar to that seen in tarsal biopsies; that is, most of the epithelial $CD3^+$ cells stained with CD45Ro (CD45Ro/CD3 = 1.1) compared to only half of the $CD3^+$ cells in the substantia propria (CD45Ro/CD3 = 0.5). An increase in the number of $HLA\text{-}DR^+$ cells in the bulbar substantia propria and in the number of $CD3^+$ cells in the bulbar epithelium were noted but these did not reach a statistically significant level.

The distribution of TCR$\gamma\delta$ was similar to that seen in tarsal tissue, with 2.2% of $CD3^+$ cells in the substantia propria positive for this antigen. Cells bearing HML-1 were present in both the epithelium and the substantia propria with a greater percentage of $CD8^+$ cells expressing this in the epithelium (47.6%) compared with the substantia propria (27.2%).

3.4 Discussion

3.4.1 Leukocytes in the normal human conjunctiva

Our results show that T cells, macrophages and occasional B cells and neutrophils in the epithelium, and T cells, B cells, macrophages, plasma cells, NK cells, mast cells and neutrophils in the substantia propria may be considered normal. Eosinophils were not seen and the presence of even a few eosinophils appears to be abnormal (Allansmith et al., 1978). T cells were the most frequent cells, in accordance with most previous work (Chan et al., 1988; Sacks et al., 1986a; Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982). The absence of plasma cells, NK cells and mast cells from the epithelium, despite their presence in the substantia propria, has been noted previously (Allansmith et al., 1978; Bernauer et al., 1993a; Irani et al., 1990).

3.4.2 Variations of leukocytes in different conjunctival areas

The cellular composition of bulbar conjunctiva was in general similar to that of tarsal conjunctiva. There were greater numbers of leukocytes, particularly $CD3^+$ and NK cells, in the bulbar region and increased IL-2R expression, which implies a greater degree of T cell activation. Allansmith (Allansmith et al., 1978) found greater numbers of leukocytes (including lymphocytes) in the tarsal conjunctiva compared with the lower fornix whereas Sacks (Sacks et al., 1986a) found the greatest number of lymphocytes in the fornix and least in the bulbar area. Variations in density and activation of lymphocytes in different conjunctival regions might relate to differences in exposure to antigen.

3.4.3 Numbers of $CD4^+$ and $CD8^+$ cells

Published normal conjunctival CD4:CD8 ratios vary, but a slight preponderance of $CD8^+$ cells has been the most frequent finding (Chan et al., 1988; Sacks et al., 1986a; Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982) which we noted in tarsal but not bulbar samples. Previous work includes a mixture of bulbar, tarsal and forniceal areas, which may influence the measured ratio, although when Sacks et al. (Sacks et al., 1986a) compared bulbar, tarsal and forniceal regions they found higher numbers of $CD8^+$ T cells in all areas. We found the CD4:CD8 ratio in the epithelium to be different from that in the substantia propria, with CD8+ cells outnumbering $CD4^+$ cells in the epithelium and a slight preponderance of $CD4^+$ cells in the substantia propria. Most previous reports show that the majority of epithelial T cells are $CD8^+$ (Chan et al., 1988; Sacks et al., 1986a; Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982; Dua et al., 1995; Soukiasian et al., 1992) with a higher CD4:CD8 ratio in the substantia propria. There may be more $CD4^+$ than $CD8^+$ cells (Chan et al., 1988; Foster et al., 1991; Dua et al., 1995) or the CD4:CD8 ratio may approximate to 1 with slightly more $CD8^+$ cells (Sacks et al., 1986a; Bernauer et al., 1993a; Bhan et al., 1982; Soukiasian et al., 1992). It has been suggested that normal CD4:CD8 ratios

may be in physiological immunoequilibrium; (Sacks et al., 1986a; Jakobiec et al., 1984), which is disturbed in inflammatory conjunctival disorders, with increased CD4:CD8 ratios in both the epithelium and substantia propria (Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982; Dua et al., 1995; Soukiasian et al., 1992; Jakobiec et al., 1984; Power et al., 1993).

The sum of $CD4^+$ and $CD8^+$ cell numbers is less than the number of $CD3^+$ cells. There are a number of possible reasons for this. There may be some double negative $CD3^+$ cells (CD-3+, CD-4-, CD-8-) as in the gut mucosa (Cerf-Bensussan and Guy-Grand, 1991; Beagley and Elson, 1992). CD3 antigen may be expressed on NK cells, but this effect is minimal as <5% of NK cells bear the CD3 molecule (Hellstrand and Hermodsson, 1990) and there were few NK cells in our specimens. A further possibility is that in a proportion of cells bearing CD4 or CD8 the density of expression is too low to be recognised by the monoclonal antibody or substrate reaction.

3.4.4 Memory T cells and activated T cells

CD45Ro is the high molecular weight isoform of CD45 and identifies memory or primed T cells (Merkenschlager and Fisher, 1991). $CD45Ro^+$ cells accounted for almost 60% of the total number of $CD3^+$ T cells and between 75 and 100% of $CD3^+$ cells in the epithelium. This suggests that the majority of normal conjunctival T cells are of the memory phenotype and have experienced antigen. Macrophages and some granulocytes may bear CD45Ro, (Frew and Kay, 1991) but probably the great majority of $CD45Ro^+$ cells were T cells, as the CD45Ro staining localised to clusters of CD3 staining and not to the more uniformly dispersed CD68 and NPE staining. The low expression of IL-2R suggests that most of these cells, though primed, were not in a current state of activation.

3.4.5 Antigen-presenting cells and HLA-DR^+ cells

MHC class II^+ conjunctival APC, involved in antigen transportation to regional lymph nodes and interaction with local lymphocytes, include macrophages and specialised APC (Langerhans cells in the conjunctival epithelium and non-Langerhans dendritic cells in the substantia propria) (Smolin, 1985; Roitt et al., 1993; Sacks et al., 1986a & 1986b; Chandler and Gilette, 1983; Chan et al., 1988; Bodaghi et al., 1994). In this study, HLA-DR staining was mainly confined to $CD68^+$ macrophages or cells with dendritic morphology in the epithelium which were considered to be Langerhans cells. A few epithelial cells also stained for HLA-DR and it has been suggested that mucosal epithelial cells may have the capacity to present antigen (Devalia and Davies, 1993).

3.4.6 CALT

The presence of scattered lymphocytes within a mucosa does not constitute MALT, that is, a specific arrangement of lymphoid tissue with substantia propria aggregates under specialised epithelium, scattered LPLs and IELs (Cerf-Bensussan and Guy-Grand, 1991; Beagley and Elson, 1992; Tomasi et al., 1980; Roitt et al., 1993) where constituent lymphocytes demonstrate mucosal recirculation, homing, and involvement in the production of sIgA (Beagley and Elson, 1992; Tomasi et al., 1980; Bienenstock and Befus, 1984). Chickens, rabbits and guinea pigs have a well-developed CALT (Franklin and Remus, 1984; Fix and Arp, 1991; Axelrod and Chandler, 1978; Latkovic, 1989), but experience with MALT in other mucosae has shown that extrapolating from animals to humans may be misleading (Cerf-Bensussan and Guy-Grand, 1991; Pabst, 1992). The human conjunctiva is known to contain lymphocytes and plasma cells which show the functional properties of MALT (Smolin, 1985; Chandler, 1996; Bloomfield, 1985).

3.4.7 Conjunctival IELs

The work presented here and elsewhere shows that conjunctival IELs are similar to gut IEL being predominantly $CD8^+$, found in the basal epithelial layers and $HML\text{-}1^+$ (Roitt et al., 1993; Cerf-Bensussan and Guy-Grand, 1991; Beagley and Elson, 1992; Bienenstock and Befus, 1984; Chan et al., 1988; Sacks et al., 1986a; Bhan et al., 1982; Jakobiec et al., 1984; Dua et al., 1994 & 1995). We have shown a very high expression of CD45Ro by IEL in the conjunctiva which parallels that seen in gut IEL (Cerf-Bensussan and Guy-Grand, 1991). However, unlike the gut, we did not find tropism of TCR$\gamma\delta$ cells to the conjunctiva, which confirms previous work (Cerf-Bensussan and Guy-Grand, 1991; Soukiasian et al., 1992) and is similar to findings in other non-gastrointestinal mucosal sites (Groh et al., 1989).

3.4.8 Conjunctival LPLs and CALT aggregates

Scattered lymphocytes in the human conjunctival substantia propria share some properties with LPLs in other MALT, with a CD4:CD8 ratio near to 1 and frequent expression of CD45Ro (Beagley and Elson, 1992; Chan et al., 1988; Sacks et al., 1986a; Bhan et al., 1982; Schieferdecker et al., 1992). It is widely stated that CALT aggregates in the conjunctival substantia propria are the norm (Chandler and Gillette, 1983; Smolin, 1985; Records, 1988; Arrfa, 1991; Streilen, 1996) and CALT aggregates lying under specialised epithelium occur in humans (Dua et al., 1994; Wotherspoon et al., 1994). However, our work and that of others (Wotherspoon et al., 1994) suggests that the presence of organised lymphoid aggregates is not universal. In the study of Wotherspoon et al. (1994), examination of the entire human superior

and inferior forniceal conjunctiva in 87 autopsy patients demonstrated organised lymphoid tissue in only 27 cases (31%). We found an aggregate in only 1 bulbar specimen but we did not examine forniceal tissue, where CALT aggregates are most likely to be found (Dua et al., 1994). It may be that the situation in the human CALT is similar to that in BALT (bronchus-associated lymphoid tissue), where over-enthusiastic extrapolation from animal work has lead to the widespread belief of the universal presence of organised MALT, whereas in fact it may be present in a minority (even if a substantial minority) of patients (Pabst, 1992; Wotherspoon et al., 1994). Whether the presence of organised CALT relates to a lifetime's history of antigenic exposure, waxes and wanes with current antigenic and immune status or is under some other, as yet unknown, control remains to be discovered.

Chapter 3: Tables and Figures

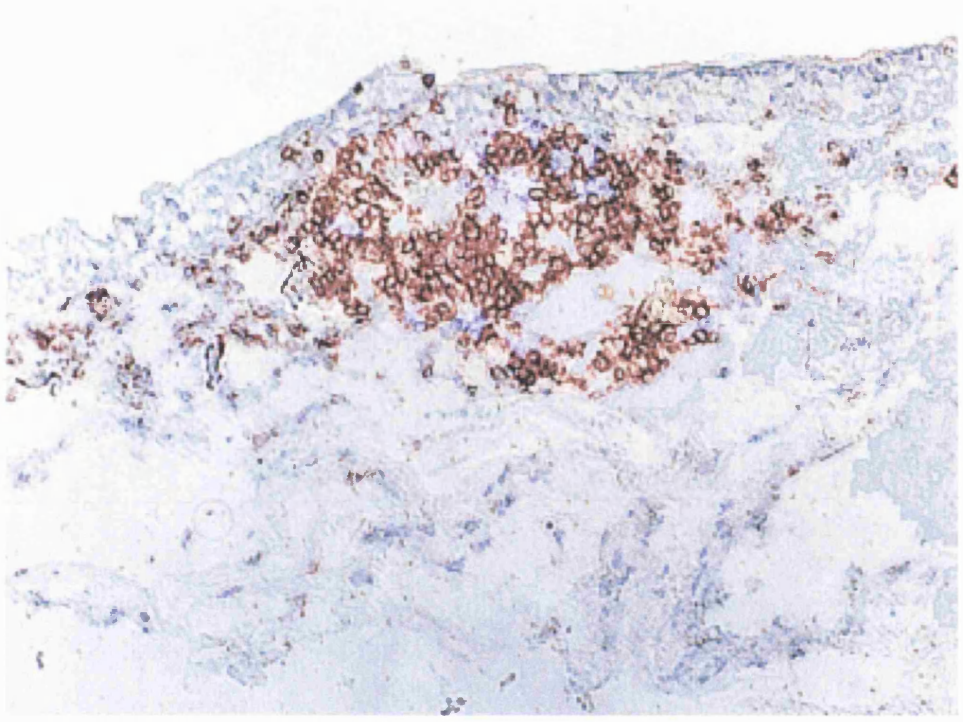


Figure 3.1. Normal bulbar conjunctiva containing a single aggregate of $CD3^+$ lymphoid cells directly under the epithelium (x100, AEC).

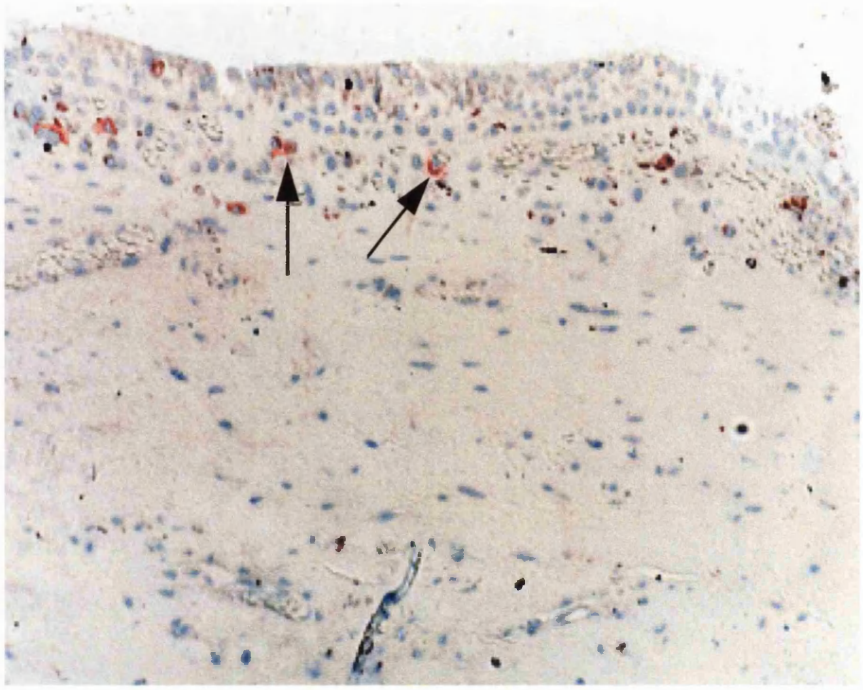


Figure 3.2. Expression of CD3 in the epithelium and substantia propria of normal tarsal conjunctiva (x100, AEC).

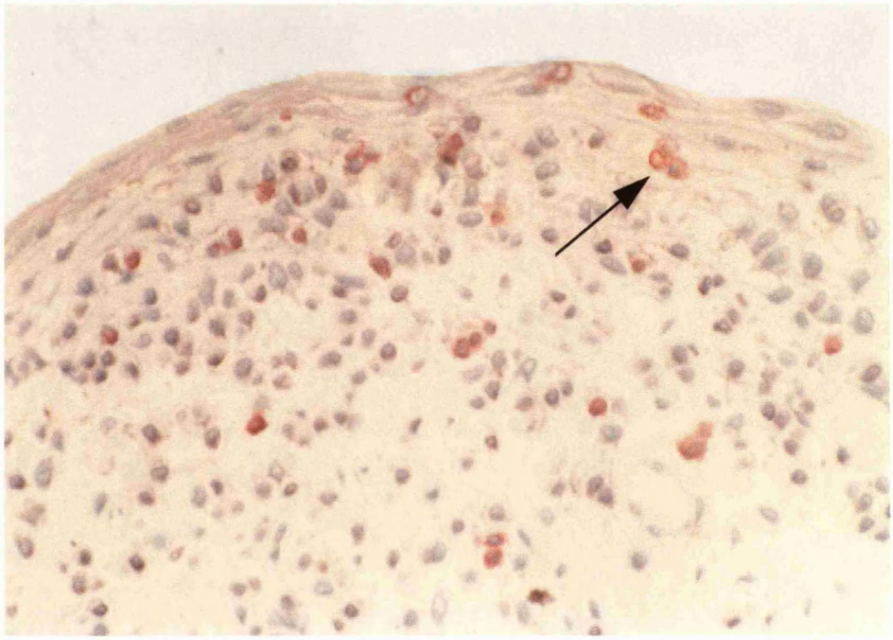


Figure 3.3. Expression of CD3 in normal bulbar conjunctiva (x200, AEC).

Table 3.1: Monoclonal antibodies used in immunohistochemical examination of normal tarsal and bulbar conjunctiva.

Monoclonal antibody	Specificity	Source
CD3	Pan T cell marker	Dako
CD45Ro	Memory T cells	Dako
CD4	Helper/inducer T cells	Dako
CD8	Suppressor/cytotoxic T cells	Dako
CD25	IL-2 receptor	Dako
TCRγδ	T cell receptor γδ	T cell Science,
Ber-ACT8	Human mucosal lymphocyte antigen-1	Dako
CD20	B cells (not plasma cells)	Dako
HLA-DR	MHC class II molecules	Dako
CD68	Macrophages, monocytes	Dako
NPE	Neutrophils (elastase)	Dako
AA-1	Mast cells	Dako
CD57	Natural killer cells	Sera-Lab
EG-1	Eosinophils (cationic protein)	Sera-Lab

Table 3.2: Cell counts in the epithelium and substantia propria of normal tarsal conjunctiva, expressed as mean count (+/-SEM) per mm of epithelium or per mm^2 substantia propria.

Cell type	Tarsal epithelium	Tarsal substantia propria
CD3 T cells	**5.3** (±1.2)	**46.7** (±9.1)
CD45Ro memory T cells	**4.0** (±0.9)	**26.6** (±6.3)
CD4 T cells	**0.6** (±0.2)	**16.0** (±4.5)
CD8 T cells	**1.8** (±0.4)	**12** (±2.5)
CD25 IL-2R	**0**	**4.2** (±1.5)
TCR-γδ	**0**	**1** (±0.9)
HML-1	**0**	2 (±1.1)
CD68 macrophage	**6.5** (±1.3)	**32.2** (±5.8)
HLA-DR MHC class II	**10.9** (±1.8)	**30.9** (±8.5)
CD20 B cell	**0**	**3.9** (±1.1)
Plasma cell	**0**	**1.1** (±0.9)
HNK natural killer	**0**	**3.9** (±1.1)
EG-1 eosinophil	**0**	0
NPE neutrophil	**0**	**2.8** (±0.2)
AA-1 Mast cell	**0**	**1.3** (±0.48)

Table 3.3: Cell counts in the epithelium and substantia propria of paired tarsal and bulbar conjunctiva, expressed as mean count (+/- SEM) per mm of epithelium or per mm^2 substantia propria using a $1mm^2$ graticule at x400 magnification. All p values are >0.1 unless stated (Wilcoxon test).

Cell type	Tarsal epithelium	Bulbar epithelium	Significance	Tarsal substantia propria	Bulbar substantia propria	Significance
CD3 T cells	**2.6** (±1.3)	**7.4** (±3.4)	**0.05<p<0.1**	**45.9** (±13.3)	**93.2** (±26.9)	**0.02<p<0.05**
CD45Ro memory T cells	**4.2** (±1.7)	**8.1** (±3.6)		**24.9** (±8.6)	**53.1** (±19.9)	**0.05<p<0.1**
CD4 T cells	**0.6** (±0.3)	**0.6** (±0.3)		**14.0** (±7.2)	**30.4** (±14.0)	
CD8 T cells	**2** (±0.8)	**2.1** (±0.9)		**16.6** (±5.0)	**14.7** (±5.7)	
CD25 IL-2R	**0**	**0.4** (±0.4)		**6.4** (±2.5)	**17.4** (±6.6)	**0.02<p<0.05**
TCR-γδ	**0**	**1** (±0.9)		**0**	**2** (±1.0)	
HML-1	**0**	**2** (±1.1)		**1** (±0.9)	**4** (±2.3)	
CD68 macrophage	**6.8** (±2.6)	**9.1** (±3.0)		**41** (±10.5)	**42.6** (±11.5)	
HLA-DR	**6.6** (±1.8)	**9.2** (±3.0)		**18.4** (±10.5)	**36.7** (±11.5)	**0.05<p<0.1**
CD20 B cell	**0.6** (±0.3)	**0**		**8** (±2.9)	**10.3** (±4.1)	
Plasma cell	**0**	**0**		**1.9** (±1.6)	**0.3** (±0.1)	
HNK natural killer	**0**	**0**		**3.4** (±1.1)	**14.3** (±5.9)	**0.01<p<0.05**
EG-1 eosinophil	**0**	**0**		**0**	**0**	
NPE neutrophil	**0**	**0.5** (±0.2)		**2.9** (±0.8)	**2.7** (±0.5)	
AA-1 mast cell	**0**	**0**		**1.9** (±0.95)	**0.5** (±0.54)	

Chapter 4

T cell cytokines in chronic allergic eye disease

4.1 Introduction

4.1.1 Evidence of the importance of T cells in chronic allergic eye disease

Certain evidence (e.g. the high frequency of atopic history, raised serum and tear IgE levels, increased tear histamine concentration and the presence of eosinophils and degranulated mast cells in the affected conjunctiva), suggest that type I hypersensitivity plays a part in these disorders (Tuft et al., 1991; Abelson and Schaefer 1993). However, the immunopathology of chronic allergic eye disease is more complex than this. A significant number of patients have no atopic history and normal IgE levels (Tuft et al., 1991; Bonini, 1993), there is marked collagen deposition (Morgan, 1971), and there is a mixed conjunctival cellular infiltrate with a strong predominance of $CD4^+$ T cells (Morgan, 1971; Foster et al., 1991; Bhan et al., 1982; Metz et al., 1996).

4.1.2 Th2-like cells are involved in non-ocular atopic disease

Variations in the cytokine profile of human $CD4^+$ T cells resembling those seen in the mouse (Mosmann and Coffman, 1989), with Th1-like cells (secreting IFN-γ) and Th2-like cells (IL-4, IL-5, IL-6 and IL-13) have been widely reported, although the situation in humans may be less clear cut (Romagnani, 1995; DelPrete et al., 1994). The biological actions of the Th2 cytokines IL-3, IL-4 and IL-5, such as the promotion of IgE production and the increased survival, activation and recruitment of eosinophils and mast cells, suggest that they are likely to control allergic inflammation (Nicola, 1994). This is supported by data from patients with atopic disorders. T cell clones derived from the peripheral blood of atopic subjects produce more IL-4 and less IFN-γ than those from normal controls (Romagnani, 1990). There is local upregulation of Th2 cytokines (and down-regulation of IFN-γ) in asthma (Hamid et al., 1991; Robinson et al., 1992 & 1993d;), AD (Kay et al., 1991), and allergic rhinitis (Durham et al., 1992) which correlates with the degree of tissue eosinophilia (Hamid et al., 1991; Robinson et al., 1993d; Kay et al., 1991; Durham et al., 1992).

4.1.3 Th subtypes in ocular allergy

Previous work had suggested that Th2 cytokines may be involved in chronic allergic eye disease. Peripheral blood T cells from patients with VKC were shown to provide significantly greater help for B cell IgE production than T cells from healthy controls (Yuasa et al., 1985). In transgenic mice, over-expression of IL-4 resulted in a blepharoconjunctivitis with histology similar to that of allergic inflammation and a raised serum IgE (Tepper et al., 1990). Romagnani (Romagnani et al., 1991; Maggi et al., 1991) showed that T cell clones derived from the conjunctiva of patients with VKC demonstrated increased production of IL-4 and decreased production of IFN-γ compared to normal controls, whilst the production of IL-2 was unchanged. These T

cells from VKC conjunctiva were better able to provide help for B cell IgE synthesis than peripheral blood T cells from the same patients (Maggi et al., 1991).

4.1.4 Objectives

This study examined T cell cytokine patterns in the conjunctiva with ISH and IHC in an attempt to establish whether distinct T cell cytokine profiles are involved in the different chronic allergic eye diseases.

4.2 Methods

4.2.1 Subjects and biopsies

3mm central superior tarsal conjunctival biopsies were obtained from 18 subjects with AKC (7 female, age 29.5 ±1.9 [21 - 46] years), 18 with VKC (5 female, age 19.4 ±1.0 [12 - 29] years) and 18 with GPC (10 female, age 38.9 ±4.2 [20 - 73] years). Specimens were also obtained from 20 non-atopic subjects (non-age-matched), with no ocular or systemic inflammatory disease. The duration of the disease was 4.7 ±0.9 years in GPC, which was significantly shorter than for VKC (8.6 ±1.2 years, p=0.009) and AKC (16.4 ±2.5 years, p=0.0001). The duration of disease in VKC was significantly less than for AKC (p=0.009).

Due to the limitations on the size of the biopsy in this area of conjunctiva, tissue samples were not large enough to allow complete analysis by both ISH and IHC. Therefore, biopsies were analysed by one technique, determined by chronology of recruitment.

Sections of biopsy for ISH were immediately snap frozen and stored at -70°C. Sections of biopsy for IHC were processed for and embedded in GMA and then stored at -20°C.

4.2.2 *In situ* hybridisation

This work was performed by Daniella Metz.

ISH using ^{35}S-labelled sense and anti-sense riboprobes for IL-2, -3, -4 and -5 was performed on 10μm cryostat sections of the conjunctival tissue to identify cytokine mRNA. Cytospins from a T cell clone known to produce IL-2, -3, -4 and -5 upon stimulation were used as positive controls. Hybridisations with sense probes or with anti-sense probes after pretreatment with RNase-A were used for negative controls. Positive cells were not seen in the negative controls.

The thickness of the sections (10μm) precluded accurate counting of positive cells. Therefore biopsies where there were clearly more than three positive cells (an arbitrary cut-off determined from this laboratory's previous experience with ISH in conjunctiva) were rated qualitatively as positive, and the number of positive biopsies in each disease group determined.

4.2.3 Immunohistochemistry

To demonstrate the presence of immunoreactive cytokine protein localising to conjunctival T cells, two-colour IHC was performed on 2μm GMA sections. IHC was first performed using a mouse anti-human cytokine monoclonal antibody (anti-IL-5 or anti-IFNγ, Table 4.1) and developed with AEC. The IHC process was repeated using mouse anti-human CD3 as the primary antibody and DAB instead of AEC. Positive and negative controls were also performed.

Cells staining positive for cytokine were identified by a red AEC stain, $CD3^+$ cells by a black stain (DAB) and T cells staining for cytokine by a brown or combined red-black colour (combination AEC and DAB) (Figure 4.1). Due to the very thin sections (2μm), detailed cell counts were possible and positive cells were counted to obtain mean counts per square mm of substantia propria. Results were expressed both as the number of samples containing double-staining T cells and as the percentage of T cells which were positive for cytokine.

4.2.4 Statistical analysis

Fisher's exact test was used to compare numbers of samples with positive staining for mRNA and the number of samples containing T cells staining positive for cytokine (two-sided p values quoted). The Mann-Whitney U test (two-sided) was used to compare the percentages of T cells which stained for cytokine protein.

4.3 Results

4.3.1 *In situ* hybridisation

ISH was performed in eight patients with AKC, eight with GPC, eight with VKC and 10 control subjects. Clear hybridisation signals were obtained with all anti-sense cytokine probes in the positive control cytospins, and negative controls revealed no specific hybridisation signal (Figures 2.7, 2.8). ISH was performed on biopsy specimens from eight patients with GPC, eight patients with AKC and eight patients with VKC and compared with 10 control specimens (Figure 4.2). The results are shown in Figure 4.3. Cytokine mRNA expression in normal tarsal conjunctiva was low but not absent. Four normal specimens revealed no signal, five revealed signal for one

cytokine and only one normal biopsy specimen hybridised with more than one cytokine probe, co-expressing IL-2, IL-3 and IL-4.

Although more AKC samples than normal tissue expressed cytokine mRNA, this did not reach statistical significance. The difference between normal (2/10 samples) and AKC (5/8 samples) IL-2 expression was the most marked. No signal was detected in three of the eight biopsy specimens from patients with AKC, but in the other five specimens there was a positive signal for IL-2 and IL-3, and in three samples all four cytokines were co-expressed.

ISH revealed significantly greater numbers of VKC samples expressing mRNA for IL-3 (7/8 vs. 3/10, p=0.04), IL-4 (7/8 vs. 2/10, p=0.01) and IL-5 (6/8 vs. 1/10, p=0.02) than normal samples, whereas there was negligible expression of IL-2 mRNA in both normal (2/10) and VKC (1/8) tissue. All VKC samples demonstrated signal for at least two cytokines. Four specimens co-expressed IL-3, IL-4 and IL-5; two co-expressed IL-3 and IL-5; and one IL-4 and IL-5. There was significantly lower expression of IL-2 mRNA in the VKC samples compared with the AKC samples (p=0.004) but there were no significant differences for the other cytokines between the two disorders.

Cytokine expression in GPC followed a similar pattern to VKC, with greater expression of IL-3 (6/8), IL-4 (6/8) and IL-5 (4/8) than normal, reaching significance for IL-4 (p=0.05). Four GPC specimens co-expressed IL-3, IL-4 and IL-5; one co-expressed IL-3 and IL-4; and only one specimen was negative for all cytokines. There was significantly greater signal for IL-2 in AKC samples (5/8) than in GPC samples (0/8, p=0.02) but otherwise no difference between the two diseases.

4.3.2 Immunohistochemistry

IHC was carried out on 10 GPC, 10 VKC, 10 AKC and 10 normal samples for IL-5 immunoreactivity to confirm ISH findings and to investigate the presence of IFN-γ. The results of immunohistochemical double-staining are shown in Figure 4.4. There were significantly more samples with T cell expression of IL-5 in VKC (8/10, p=0.004), GPC (7/10, p=0.02) and AKC (6/10, p=0.04) than normal (1/10). The percentage of T cells staining positive for IL-5 protein was lower in control samples (mean ±SEM 1.2 ±1.2) than in VKC (3.8 ±1.1, p=0.01), GPC (3.7 ±1.6, p=0.02) and AKC (5.8 ±3.0, p=0.04).

There was more marked T cell expression of IFN-γ in AKC than in normal control samples or samples from patients with the other disorders. Eight of the 10 AKC

biopsy specimens contained T cells staining positive for IFN-γ protein compared with two of 10 controls (p=0.021), four of 10 GPC and five of 10 VKC biopsy specimens. The percentage of T cells staining positive for IFN-γ was markedly increased in AKC (12.3±3.5) compared with GPC (0.6±0.3,p=0.01) and VKC (0.6±0.2, p=0.01).

4.4 Discussion

4.4.1 Techniques used

Biopsies were obtained from the superior tarsal conjunctiva in all patient groups, including AKC. This is partly because the tissue is easier to handle and orient than bulbar tissue and more importantly because this is the area of conjunctiva with maximal clinical inflammation. It has been widely quoted that the maximally inflamed area in subjects with AKC is the inferior tarsal conjunctiva (e.g. Friedlander, 1993), but this is not the experience in the United Kingdom (Tuft et al., 1991; Jay, 1981) nor for other groups (Foster, 1995; Akova et al., 1994). ISH was performed on frozen tissue but the relatively thick sections required for this technique precluded accurate cell counting because of the overlay of positive cells. Therefore, for ISH, only a qualitative (positive or negative) result was possible. To obtain quantitative results for more robust statistical analysis, we used resin embedding for IHC, which permits sectioning thin enough for very accurate cell counting (Britten et al., 1993). It is not so far possible to perform ISH on such thin resin sections.

4.4.2 Th subsets vary in the different disorders

Previous studies which had suggested that Th2 cytokines may be involved in the pathogenesis of VKC (Yuasa et al., 1985; Romagnani et al., 1991; Maggi et al., 1991) examined *in vitro*-stimulated T cells, the phenotype and function of which might be altered by such manipulation, whereas this study examines the T cell *in situ* and thus might be considered more representative of true pathophysiology. The results of the present study suggest that there are distinct differences in the profiles of T cell cytokine expression of different chronic allergic eye disorders. There is greater production of IL-3, IL-4 and IL-5 by conjunctival T cells in VKC and greater production of IL-4 and probably IL-3 (near significance of results)by conjunctival T cells in GPC than in the normal conjunctiva, a Th2-like cytokine pattern. Conjunctival T cells in AKC differ in that there is a slightly lower production of IL-3, -4 and -5 and markedly greater production of IL-2 and IFN-γ. There may be mixed populations of Th1 and Th2 cell types in AKC with a predominance of Th1 or individual AKC T cells may produce a wider range of cytokines, resembling murine Th0 cells. A number of AKC specimens expressed mRNA for IL-2, IL-3, IL-4 and IL-5 and it is uncertain to what extent they were produced by different T cell subsets.

4.4.3 Relationship to phenotypic T cell differences

Previous studies in our laboratory have shown other differences in T cells in allergic ocular disease (Metz et al., 1996). In VKC and GPC, up to 50% of $CD4^+$ T cells co-express CD45Ro and CD45Ra, whereas $CD4^+$ T cells in AKC are predominantly $CD45Ro^+CD45Ra^-$. These CD45Ro/Ra double-positive T cells may represent cells in the process of switching from a naive to a memory phenotype on antigenic stimulation or may identify a functionally distinct group of memory T cells. Interestingly, in the murine system, a correlation has been described between CD45Ro/Ra double-positive T cells and the Th2 subset (Luqman et al., 1991).

4.4.4 Relationship to other cytokine-producing cells

The results of ISH alone did not allow identification of the cell type containing the transcript, and at least some of the transcript is likely to have been in cells other than T cells (e.g. mast cells, eosinophils). The use of IHC demonstrated immunoreactive cytokine protein co-localising with $CD3^+$ cells and, although some of this may be due to cytokine released from non-T cells bound to T cell surface cytokine receptors, the IHC data correlated well with the ISH findings. Other cells, such as mast cells and eosinophils, are known to be capable of cytokine production in allergic and other types of disorders (Dubucquoi et al., 1994; Broide et al., 1992; Bradding et al., 1993), and the conjunctiva in chronic allergic eye disease is densely infiltrated by such cells. Although the numeric predominance of T cells in the tissue suggests great importance for their cytokines in the direct control of the inflammatory response, T cell-generated cytokines will also influence release of cytokine by other leukocytes, producing wider effects (including feedback on T cells) indirectly, to create a complex network of cytokine cross-talk. It is uncertain whether, like T cells, other infiltrating leukocytes will also demonstrate characteristic cytokine profiles in the different clinical disorders.

4.4.5 Possible influences on Th subsets

We do not know whether these T cell variations are a cause or an effect of different characteristics, such as chronicity and age of onset, of the various ocular allergic disorders. The same causal agent can give rise to either Th2- or Th1-like responses (e.g. leishmaniasis, leprosy) and this determines clinical characteristics and disease outcome (Kemp et al., 1994; Golding and Scott, 1995). A number of factors may influence the direction of T cell differentiation in these cases, such as the nature and amount of antigen, the type of APC, hormones, cytokines and the genetic background of the host (Romagnani, 1995; Hsieh et al., 1995). The time-scale of the disorder is a further possible influence. There is evidence that the type of Th response to a single

agent (bacillus Calmette-Guerin antigen) can vary with time (Sander et al., 1995) and the shift in the T cell cytokine profile from Th2-like to Th1-like in AKC may be a potential function of disease chronicity. The disease duration in AKC in this study was significantly longer than in VKC and GPC. The disease duration in AKC is generally much longer than in VKC, with many cases of VKC resolving spontaneously in two to 10 years (Buckley, 1989). Because of the necessity of co-operation in harvesting a conjunctival biopsy specimen, younger patients with VKC could not be included in this study and so our VKC patients are likely to be a selected group with a longer mean disease duration than in an unselected group.

The T cell differences between AKC and VKC may also be influenced by differences in steroid treatment. AKC patients usually receive more steroid drops and this hold in our group of patients, in which 13 out of 18 AKC patients had been receiving topical steroids before the washout period compared with 7 out of 18 VKC patients. However, any change due to greater steroid drop use in the AKC group would be expected to be in the opposite direction to our results, as corticosteroids generally favour the production of type 2, and suppress type 1, cytokines (Norbiato et al., 1997).

4.4.6 Relevance to clinical practice

Whether the variations in T cell function can be directly related to clinical characteristics, such as the greater conjunctival cicatrisation or the insidious nature of the corneal disease in AKC compared with other chronic allergic eye diseases, remains to be seen. These functional differences may direct research into future therapies. In the short term, it is possible, given the differences in IL-2 production, that the effects of topical CsA may prove more dramatic in AKC than they already have in VKC (Secchi et al., 1990).

4.4.7 Conclusion

In conclusion, using IHC and ISH, this study has demonstrated different functional T cell subsets in the different chronic allergic eye diseases.

Chapter 4: Tables and figures

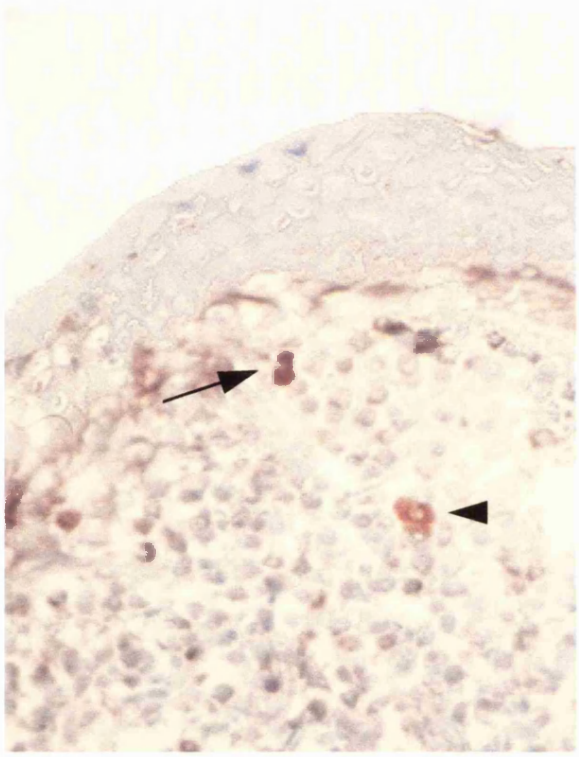


Figure 4.1a. Upper tarsal conjunctiva in VKC demonstrates cell with immunoreactivity for both CD3 (DAB) and IL-5 (AEC) (arrow) and cell with immunoreactivity for only IL-5 (arrowhead) (x200).

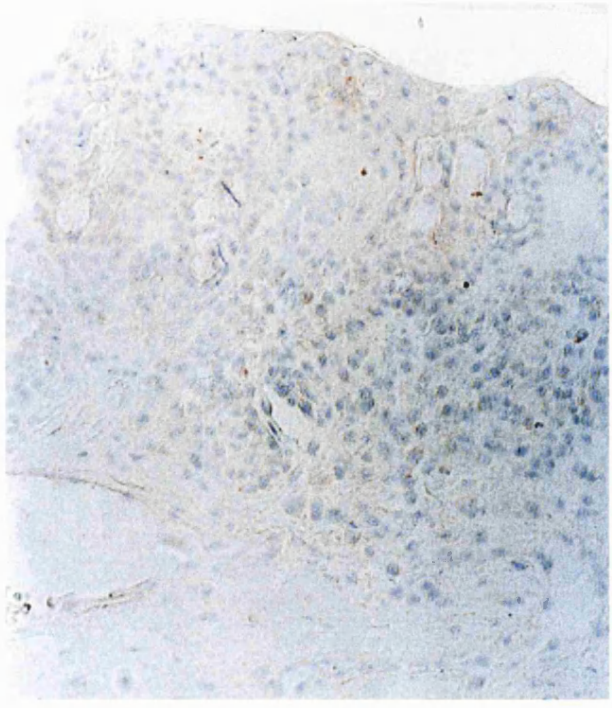


Figure 4.1b. Negative control demonstrates no positive immunostaining (x100, AEC & DAB).

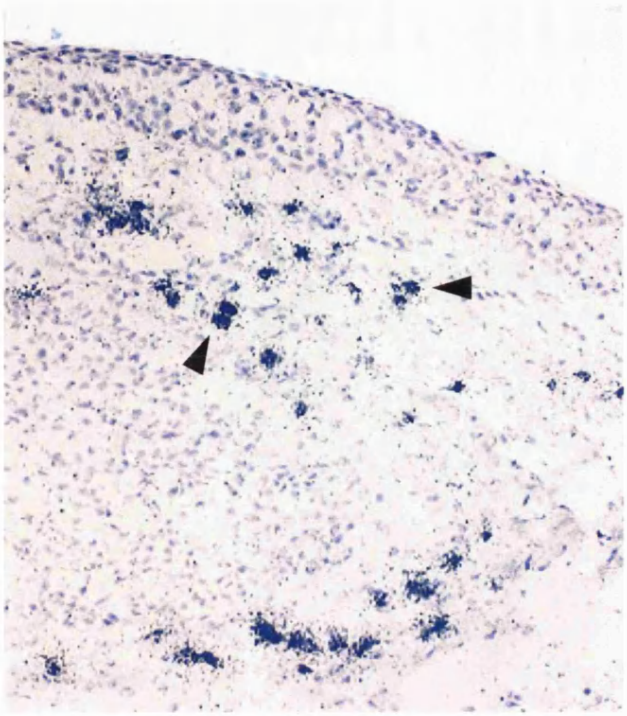


Figure 4.2a. Multiple foci of IL-2 mRNA localisation (arrowheads) in upper tarsal conjunctiva of AKC (x100).

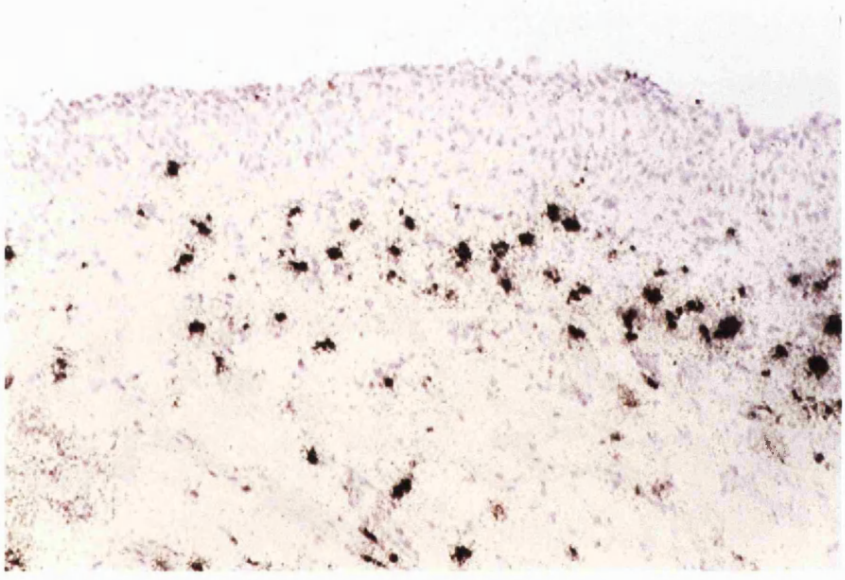


Figure 4.2b. Foci of IL-5 mRNA localisation in VKC conjunctiva (x100).

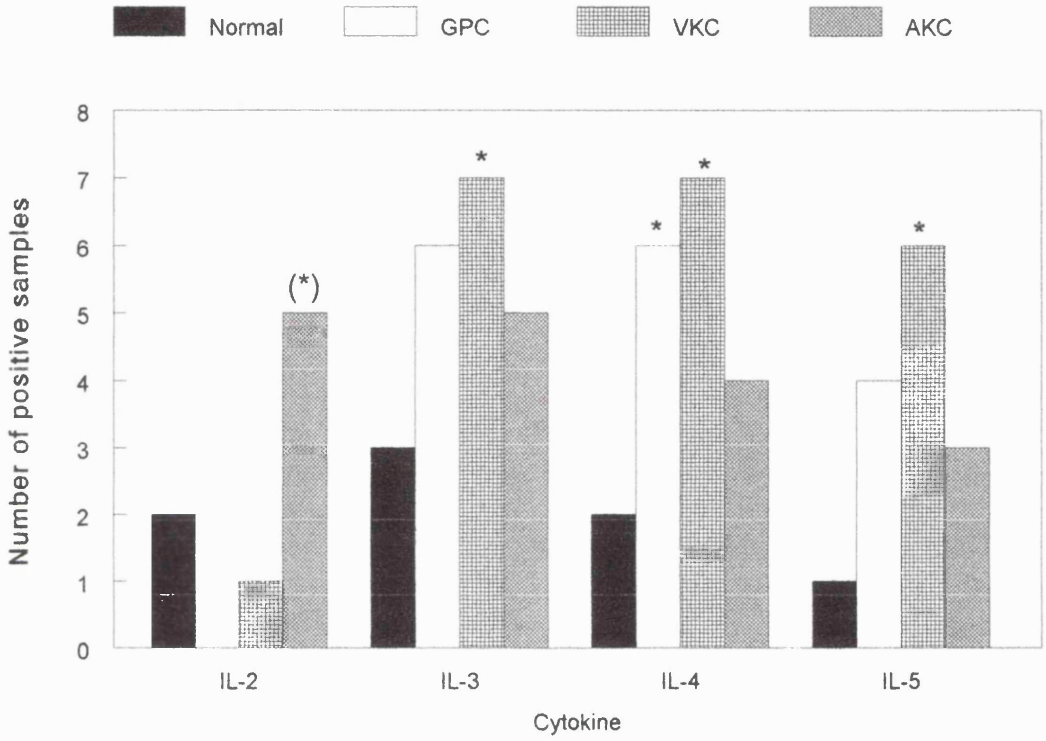


Figure 4.3. Number of samples expressing cytokine mRNA by ISH in chronic allergic eye disease (* = significant difference vs. normal controls; (*) = significant difference vs. other disorders).

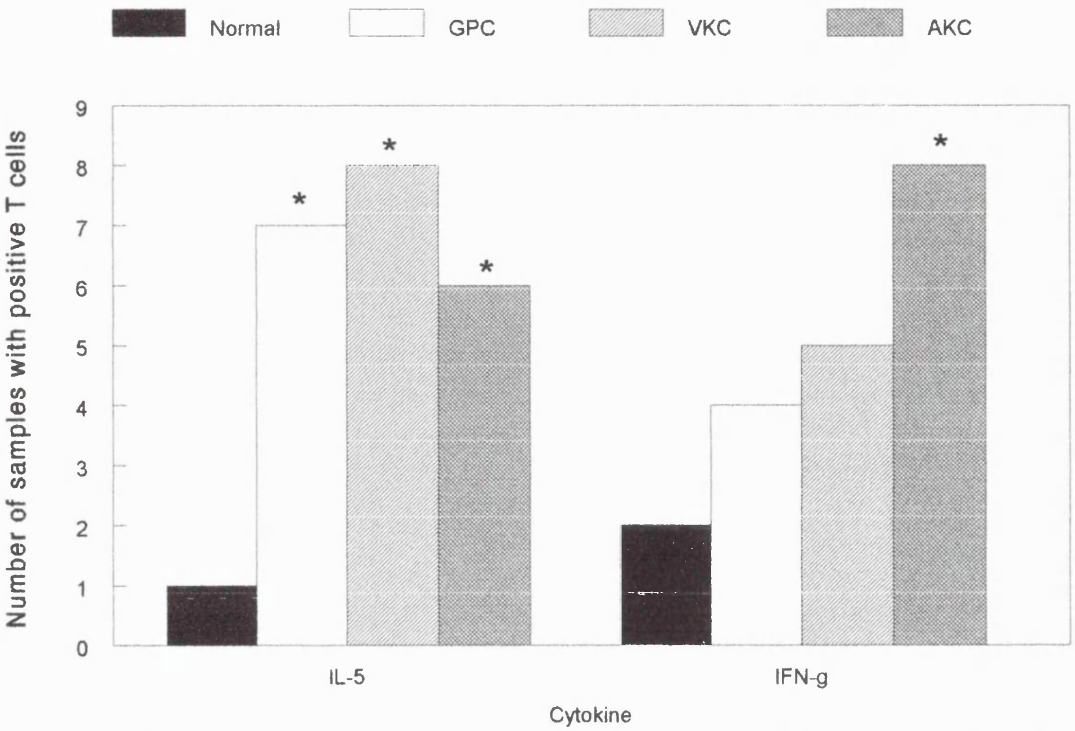


Figure 4.4a. Number of samples containing cells with co-localisation of both antigens.

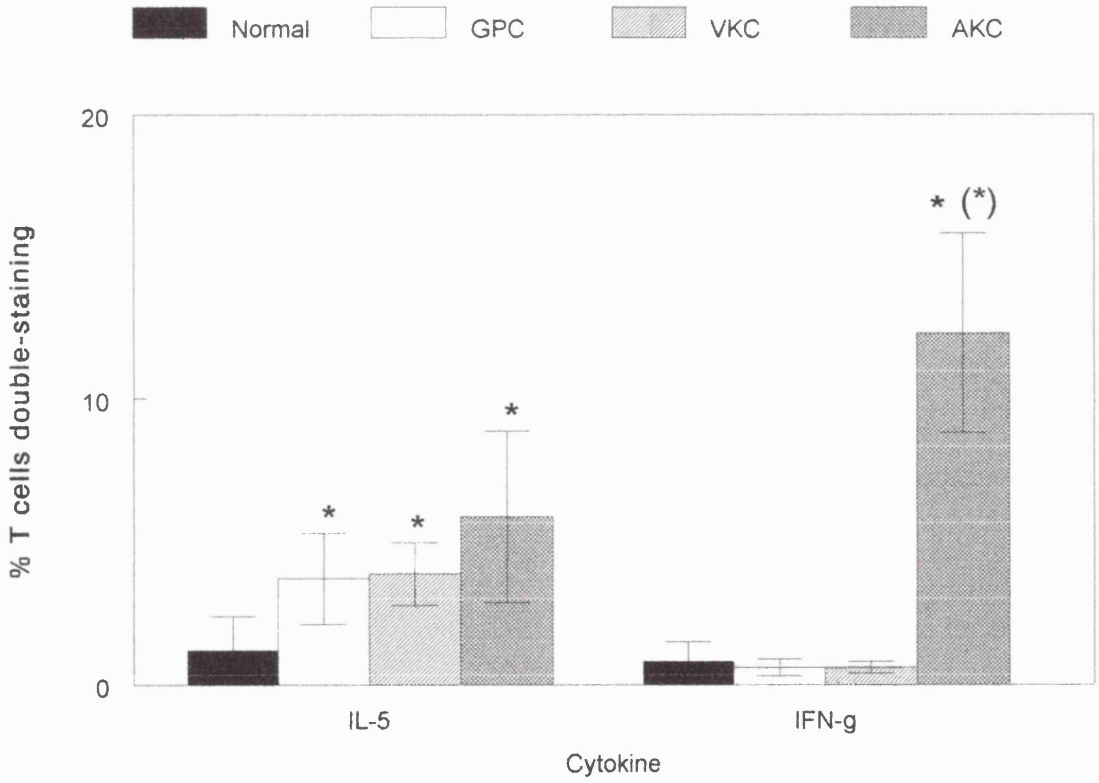


Figure 4.4b. Percentage of $CD3^+$ cells in which co-localisation of cytokine was observed.

Figure 4.4. Co-localisation of CD3 and cytokine by IHC in chronic allergic eye disease (* = significant difference vs normal; (*) = vs. other disorders)

Table 4.1: Primary monoclonal antibodies used in immunohistochemical examination of T cells and their cytokines.

Monoclonal antibody	Specificity	Source
IL-5	Interleukin-5	Genzyme
IFN-γ	Interferon-gamma	Genzyme.
CD3	Pan T cell marker	Dako

Chapter 5

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

5.1 Introduction

5.1.1 The pathophysiology of chronic ocular allergy

The pathophysiology of AKC, VKC and GPC is complex, and involves both type I hypersensitivity-like features and T cell-mediated inflammation, with a Th-2-like pattern of cytokine production in VKC and GPC and a Th-1-like pattern in AKC (Chapter 4). Cytokine production by non-T cells (e.g. mast cells, epithelium) has been implicated in non-ocular allergic disease (Bradding et al., 1993; Devalia and Davies, 1993) and recent work shows that such cytokine production is possible in the conjunctiva (MacLeod et al., 1996; Grabner et al., 1985; Schreiner et al., 1985; Jones et al., 1994).

5.1.2 Eosinophils in ocular allergy

An eosinophil infiltrate is typical and virtually pathognomonic of ocular allergy (Abelson and Schaefer, 1993). Eosinophils are able to produce a wide range of cytokines which may influence their own activity and that of other leukocytes and have been shown to do so in a number of disorders, including non-ocular atopic disorders (Hansel et al., 1993; Moqbel et al., 1994; Hamid et al., 1992b; Melanie et al., 1993; Yousefi et al., 1995; Costa et al., 1993; Walz et al., 1994; Lim et al., 1995; Wong et al., 1990 & 1991; Weller et al., 1993; Ying et al., 1993; Saito et al., 1994; Kay et al., 1995; Howarth et al., 1995; Finotto et al., 1994; Elovic et al., 1994; Ohno et al., 1992; Broide et al., 1992; Bradding et al., 1994; Tanaka et al., 1994; Desremaux et al., 1992 & 1995; Dubucquoi et al., 1994; Tan et al., 1993; Kadin et al., 1993; Lamkhioued et al., 1995). Eosinophils are also able to produce multiple inflammatory mediators, including phospholipase A2 products, toxic oxygen metabolites and granule proteins (Wardlaw and Kay, 1987). A number of lines of evidence support a link between eosinophils and the development of allergic corneal disease (Trocme et al., 1989b; Santos et al., 1994). The levels of granule proteins in tears and serum correlate with disease severity (Secchi et al., 1995; Tomassini et al., 1994) and tissue deposition occurs in areas of keratopathy (Trocme et al., 1993). These proteins do have some toxic effect on corneal epithelium (Trocme et al., 1994) but their presence may simply be a reflection of intense eosinophil activity and not the primary cause of the associated corneal disease.

5.1.3 Objectives

The aims of this study were: firstly, to perform a descriptive study to identify which cytokines are produced by eosinophils in serious ocular allergy, about which almost nothing is known, and to determine whether eosinophils, like T cells, show differences in cytokine profiles in the different disorders; secondly, to investigate the hypothesis

that eosinophils play a highly important role in the pathogenesis of corneal disease in ocular allergy. Therefore, an examination of 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 which affect the cornea (AKC, VKC) and those that do not (GPC) was conducted. Cytokines were chosen which are known to be associated with eosinophils in other tissues.

5.2 Methods

5.2.1 Subjects and biopsies

Central superior tarsal conjunctival biopsies were obtained from subjects with VKC (n=10, 2 female, age 20.0 ±1.6 [12-29] years), GPC (n=10, 3 female, 41.8 ±6.3 [20-73] years) and AKC (n=10, 3 female, 27.9 ±2.3 [21-46] years). All subjects with VKC or AKC had current or previous corneal involvement. Biopsies were obtained from 10 normal, non-age-matched, non-atopic controls (6 female, age 55.2 ±7.3 [19-85] years). The biopsies were processed for, and embedded in, GMA resin.

5.2.2 Immunohistochemistry

IHC was performed on 2μm sections. For cell counts, one-colour IHC was performed using appropriate primary antibodies (Table 5.1) with AEC as chromagen. For the localisation of antigens and cytokines to eosinophils, two-colour IHC was performed, using AEC as the chromagen to detect antigen or cytokine immunoreactivity and DAB as the chromagen to detect ECP immunoreactivity. Appropriate positive and negative controls were performed (Figure 5.1). Haematoxylin and eosin (H&E) staining was employed for morphological definition and orientation of the sections and to identify plasma cells.

5.2.3 Cell counts and statistical analysis

Cells staining positively for cell identification markers (e.g. CD20 for B cells), cytokines and eosinophil activation markers were identified by a red AEC reaction product (Figure 5.1). Plasma cells were identified by morphology on H&E sections. Eosinophils were identified by the black-staining DAB reaction product, and eosinophils which stained for cytokines or cell surface antigens by a brown or red-black colour due to the combination of AEC and DAB (Figure 5.2).

Masked cell counts were performed and expressed as mean counts per square mm of substantia propria. Individual leukocyte counts were summed for a total leukocyte count to allow calculation of the percentage of leukocytes which were eosinophils. For

eosinophil double-staining, the results were expressed as the number of samples exhibiting any double-staining and the percentage of eosinophils in each sample which were double-stained.

A non-parametric statistical test (Mann-Whitney U test, two-tailed) was employed to analyse the differences in cell numbers and percentages between the disease groups, and Fisher's exact test (two-tailed) to analyse differences in the numbers of samples.

5.3 Results

5.3.1 Cell counts (Table 5.2)

There were very few eosinophils in normal human tarsal conjunctiva but there was marked eosinophil infiltration in all the diseased tissues examined, with significantly greater numbers of eosinophils in all the disorders than in normal subjects (Figure 5.3). The eosinophil infiltrate was more dense in GPC (mean ± SEM 21.6±8.2/mm^2) and VKC (16.2±4.3/mm^2) than in AKC (3.6±0.9/mm^2) and these differences were statistically significant (p=0.002, p=0.009 respectively). Eosinophils constituted a greater percentage of infiltrating leukocytes in the allergic tissue (GPC 11.1±1.3 p=0.002, VKC 11.5±2.5 p=0.0003, AKC 3.6±0.9 p=0.007) than in normal tissue (0.5± 0.2) . Eosinophils accounted for a greater percentage of conjunctival leukocytes in GPC (p=0.001) and VKC (p=0.01) than in AKC.

5.3.2 Eosinophil cell surface antigens

The results of cell surface antigen staining for eosinophils is shown in Figure 5.4. 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 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: 5/10 VKC, 6/10 AKC, 2/10 GPC; HLA-DR: 7/10 VKC, 8/10 AKC, 3/10 GPC; ICAM-1: 8/10 VKC, 7/10 AKC, 3/10 GPC; IL-2R: 6/10 VKC, 6/10 AKC, 0/10 GPC). The percentages of tissue eosinophils staining for surface antigen in each sample were also higher in VKC and AKC than GPC for all cell surface antigens (mean ±SEM: CD4: 10.7 ±4.2% VKC, 24.9 ±7.5% AKC, 1.8 ±1.3% GPC; HLA-DR: 24.9 ±6.3% VKC, 44.4 ±9.4% AKC, 10.1 ±5.5% GPC; ICAM-1: 38.6 ±8.9% VKC, 24.7 ±5.8% AKC, 4.6 ±2.6% GPC; IL-2R: 8.6 ±2.6% VKC, 21.2 ± 6.7% AKC, 0% GPC) and this reached statistical significance in AKC for CD4 (p=0.03), HLA-DR (p=0.01), ICAM-1 (p=0.01) and IL-2R (p<0.0001) and in VKC

for ICAM-1 ($p=0.07$) and IL-2R ($p<0.0001$). IL-2R staining was seen only on eosinophils in VKC and AKC. There were no significant differences between AKC and VKC.

In the pathological specimens in which $CD4^+$ eosinophils were present, they constituted 20.3 ±4.2% (range 2.9-44.4%) all $CD4^+$ cells. This did not vary significantly between the different clinical disorders: 24.1 ±16% GPC, 17.1 ±7.2% VKC, 21.7 ±5.9% AKC.

5.3.3 Cytokine localisation to eosinophils

Results for each disorder are given as mean ±SEM percentage of eosinophils with positive cytokine staining (%eos) and number of samples (out of 10) containing eosinophils with positive cytokine staining (Figure 5.5). There were very few eosinophils in normal conjunctival samples, but in none of these could cytokine co-localisation be demonstrated. A number of cytokines localised to eosinophils in all the allergic eye disorders to a similar degree; these were RANTES (VKC 9.7 ±5.0% eos, 3/10 samples; AKC 11.6 ±6.1% eos, 3/10 samples; GPC 1.3 ±1.3% eos, 1/10 samples), TGF-β (VKC 18.7 ±8.5% eos, 4/10 samples; AKC 21.8 ±7.7% eos, 5/10 samples; GPC 11.7 ±6.6% eos, 3/10 samples) and TNF-α (VKC 28.7 ±8.8% eos, 7/10 samples; AKC 22.4 ±7.7% eos, 5/10 samples; GPC 18.0 ±7.4% eos, 6/10 samples). Certain cytokines were localised to eosinophils only in certain disorders. Eosinophil IL-3 was observed in VKC (22.5 ±6.0% eos, 7/10 samples) and only minimally in AKC (1.5 ±1.5% eos $p=0.006$, 1/10 samples $p=0.02$) and not in GPC ($p=0.002$). Eosinophil IL-5 occurred in GPC (7.5 ±3.6% eos, 4/10 samples) and VKC (18.5 ±5.5% eos, 6/10 samples) only, to similar extents.

Eosinophil IL-6 was greater in VKC (17.0 ±7.3% eos, 5/10 samples) than in GPC (0.42 ±0.42% eos $p=0.04$, 1/10 samples) and also greater (but not reaching statistical significance) than AKC (5.3 ±3.9% eos, 2/10 samples). IL-4 co-localisation was seen in all the disease groups but was significantly greater in AKC (41.1 ±9.3% eos, 9/10 samples) than GPC (4.3 ±2.5% eos $p=0.001$, 3/10 samples $p=0.02$) and VKC (5.7 ± 2.5% eos $p=0.002$, 4/10 samples $p=0.04$). GM-CSF co-localisation was much greater in both AKC (29.8 ±8.4% eos $p=0.01$, 6/10 samples $p=0.04$) and VKC (21.8 ±6.9% eos $p=0.01$, 6/10 samples, $p= 0.04$) than GPC (1.5 ±1.5% eos, 1/10 samples). Interleukin-8 co-localisation was greater in AKC (36.6 ±10.7% eos, 7/10 samples) than VKC (2.4 ±1.6% eos $p=0.009$, 2/10 samples $p=0.06$)and GPC (10.7 ±7.2% eos, $p=0.05$, 2/10 samples $p=0.06$).

5.4 Discussion

5.4.1 Techniques used in the study

The use of GMA resin allowed ultra-thin sectioning and very accurate cell counting. A choice was made to use monoclonal antibodies against specific eosinophil granule proteins for eosinophil counts and localisation of antigens to eosinophils, rather than identify eosinophils by their characteristic morphology. This is because this morphology may be lost or obscured in degranulated or degranulating cells (Gleich and Adolphson, 1986), leading to under-identification of eosinophils. The use of IHC for the study of eosinophil cytokines is acceptable as, like mast cells, they contain significant amounts of stored cytokine. In comparison, T cell work relies more heavily on detection of mRNA by ISH, as T cell cytokines are released almost immediately (Bradding et al., 1993).

5.4.2 Eosinophil activation in ocular allergy

It is believed that eosinophils may be central to the pathogenesis of allergic keratopathy (Trocme et al., 1989b; Santos et al., 1994). 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, the study 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 (Hansel et al., 1991; Hansel et al., 1992; Czech et al., 1993; Plumas et al., 1991; Sakamoto et al., 1996; Kroegel et al., 1992). The results showed that there was increased expression of such antigens (CD4, HLA-DR, ICAM-1, IL-2R) in those disorders which 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, which is important in the development of corneal disease, via greater release of epitheliotoxic mediators and cytokines from activated eosinophils.

5.4.3 Eosinophil cytokines in ocular allergy

The conventional view of the eosinophil as a simple effector leukocyte in allergic inflammation is no longer tenable as eosinophils are, via their production of a wide range of cytokines, likely to orchestrate inflammation in atopic and other diseases (Venge and Hakansson, 1991). However, we do not yet know the pattern and range of tissue eosinophil cytokine production in any one disorder nor whether the spectrum

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

The results of this study suggests that eosinophils are an important source of cytokines in serious ocular allergic disease and show the spectrum of cytokines localising to eosinophils differs in the different disorders, although it is accepted that the technique does not measure cytokine release nor prove cytokine synthesis, which requires ISH (work to commence shortly).

5.4.4 The relationship between variations in eosinophil activation, cytokine content and the different disorders

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, which 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 (Pazdrak et al., 1997; Sanderson, 1995). 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 be releasing their cytokines and could be playing 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 pro-inflammatory, can be chemoattractant for other cell types (mast cells, B cells etc.) and this would in turn lead to other cytokines being produced within the tissue (Lindemann and Mertelsmann, 1995; Barton, 1996; Steward, 1993). In AKC there is expression of IL-4, IL-8 and GM-CSF. IL-4 is not only a B cell stimulator but can influence the development of T cells towards a Th2-like phenotype (Puri, 1995). Although mast cells are known to synthesise and secrete IL-4 in ocular allergy (MacLeod et al., 1995),

the role of eosinophils in producing IL-4 in AKC has not previously been demonstrated. IL-8 is a potent neutrophil chemoattractant (Baggiolini et al., 1994) and therefore, in AKC, the activated eosinophils are likely to be contributing to a wider variety of pathways, which could be involved in the chronicity and corneal involvement observed. It remains to be seen if the common cytokine detected in VKC and AKC eosinophils (GM-CSF) is the one which dictates whether corneal involvement occurs.

5.4.5 Possible influences on the eosinophil variations

Although other cells (T cells, mast cells) are present in the inflammatory infiltrate, the results in this study support an important role for eosinophils in the pathogenesis of disease. The previous study (Chapter 4) has shown functionally distinct T cell subsets in the different chronic ocular allergic diseases. 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 which could itself influence T cell behaviour. 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 cicatrisation (much greater in AKC than VKC). None of the cytokines were positive in all cases in any disease group which may be related to inter-patient differences in clinical characteristics.

5.4.6 Conclusion

Elucidation of eosinophil function is likely to be critical for the understanding of ocular, and other, allergic disease. This study has begun the characterisation of eosinophil mechanisms in detail. Similar studies in other tissues will allow comparison with asthma, rhinitis and AD.

Chapter 5: Tables and figures

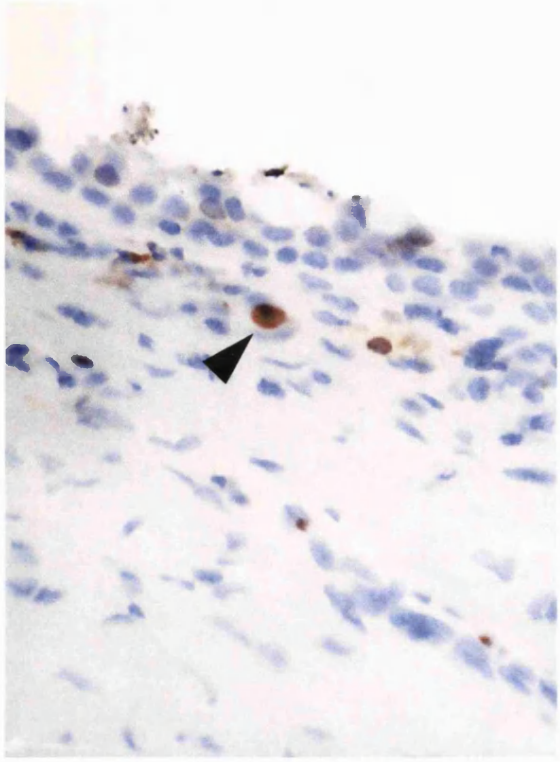


Figure 5.1a. Red AEC deposition demonstrating IL-4 immunoreactivity (x200).

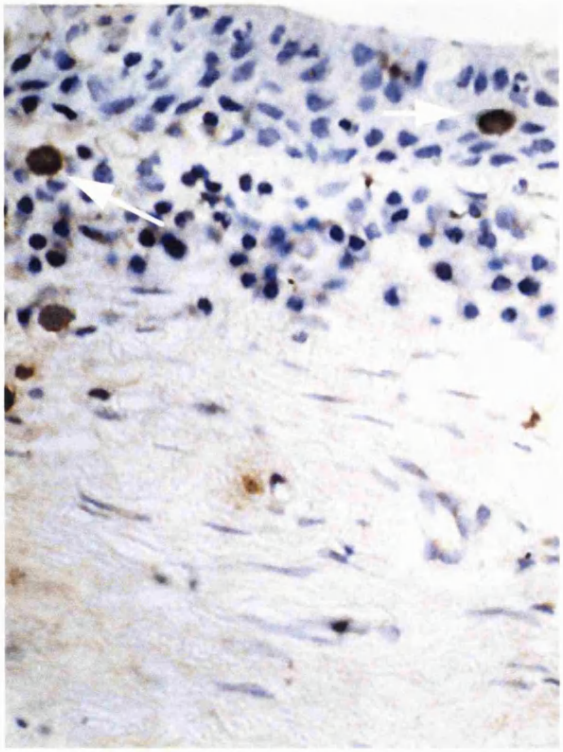


Figure 5.1c. Black DAB single positive cells: ECP^{+} eosinophils (x200).

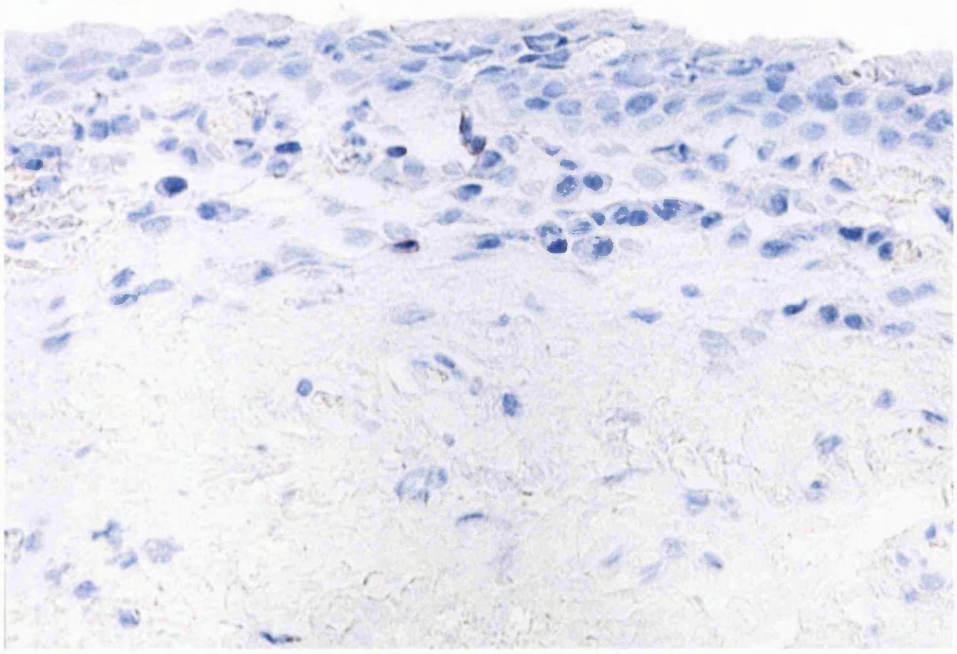


Figure 5.1b. No immunoreactivity in control using unrelated antibody (x200).

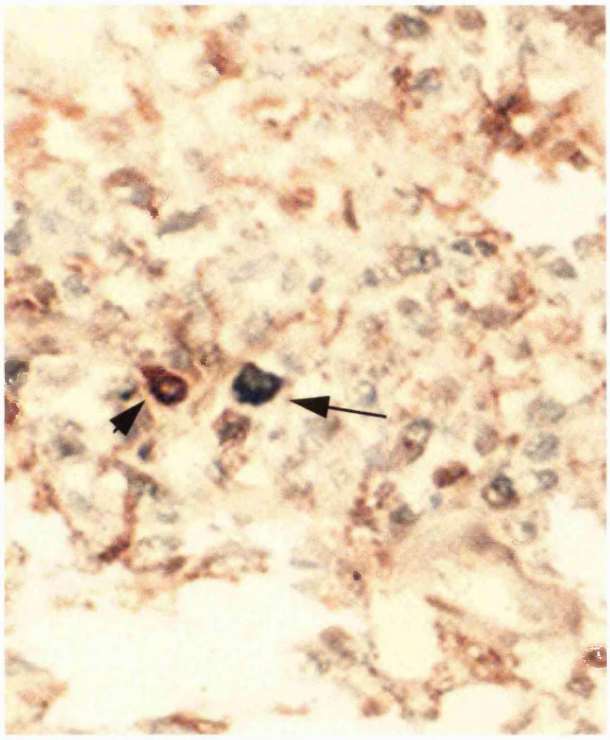


Figure 5.2a. Example of DAB single positive cell and AEC-DAB double positive cell: an eosinophil stains black (ECP^+, DAB, black arrow); an eosinophil expressing IL-5 (AEC) shows a combined red and black stain (arrowhead) (x200).

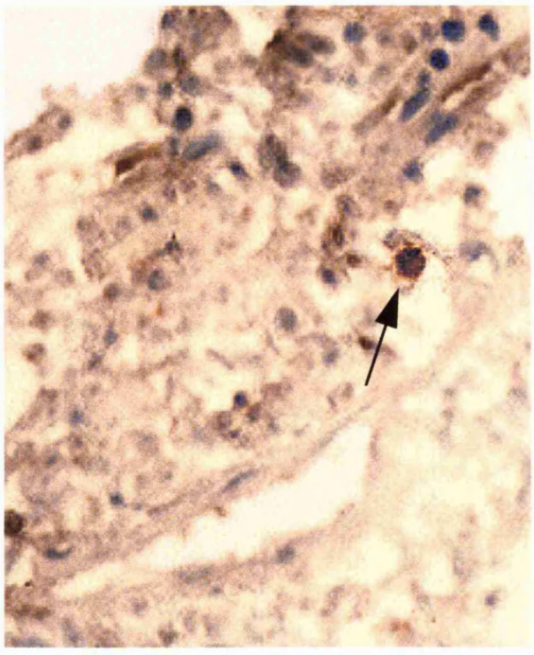


Figure 5.2b. Example of AEC-DAB double-positive cell, an eosinophil expressing ICAM-1 (x200)

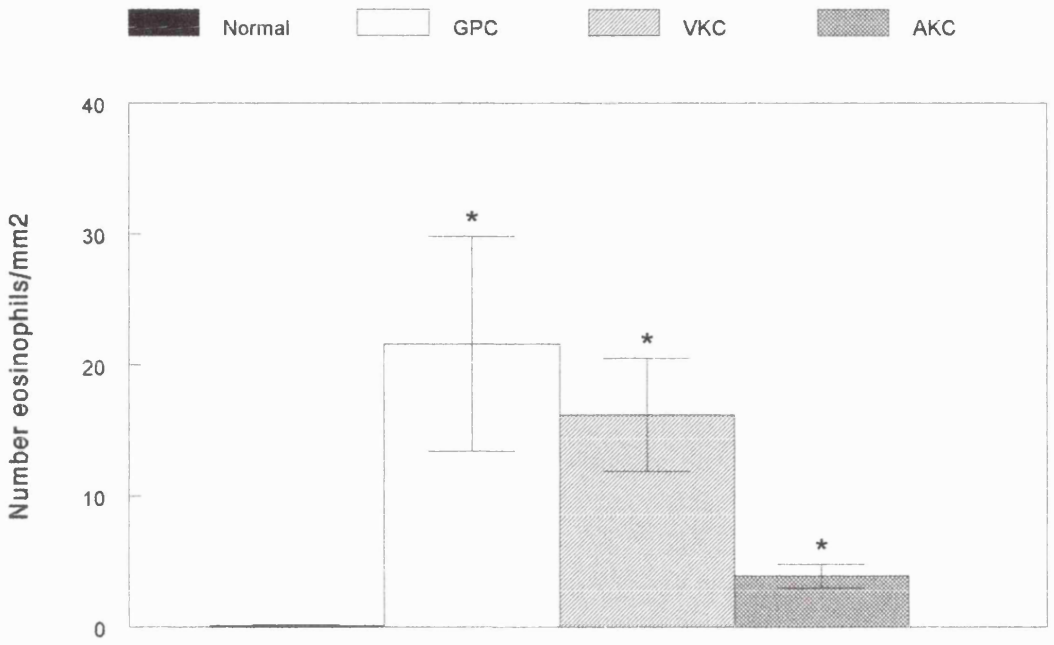


Figure 5.3a. Numbers of eosinophils per mm^2 substantia propria.

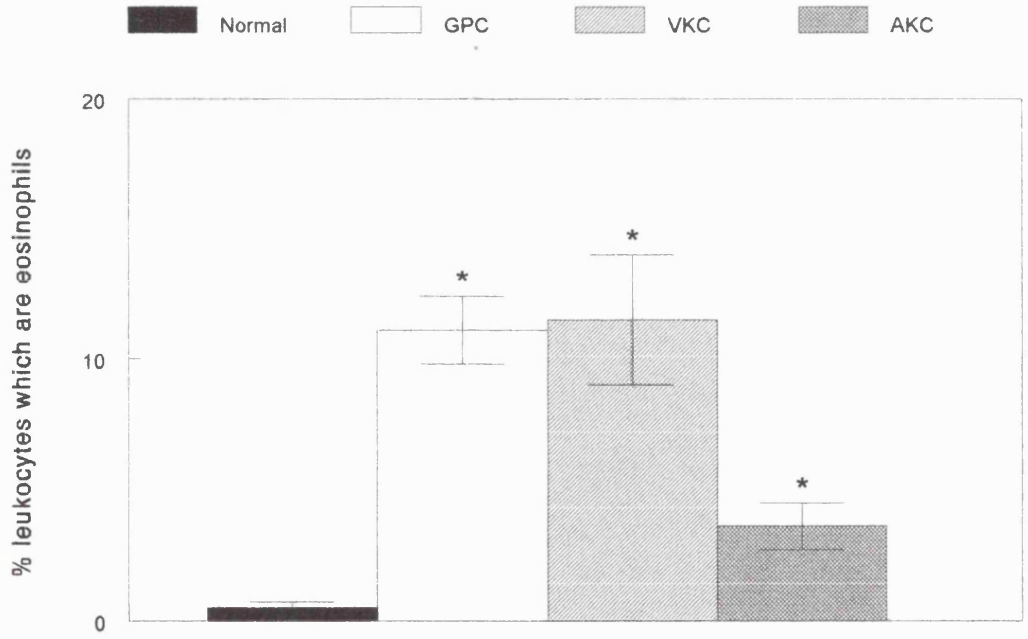


Figure 5.3b. Eosinophils as a percentage of the total number of infiltrating leukocytes.

Figure 5.3. Numbers (mean, SEM) of eosinophils in the substantia propria in normal and allergic conjunctiva
(* = significant difference vs. normal).

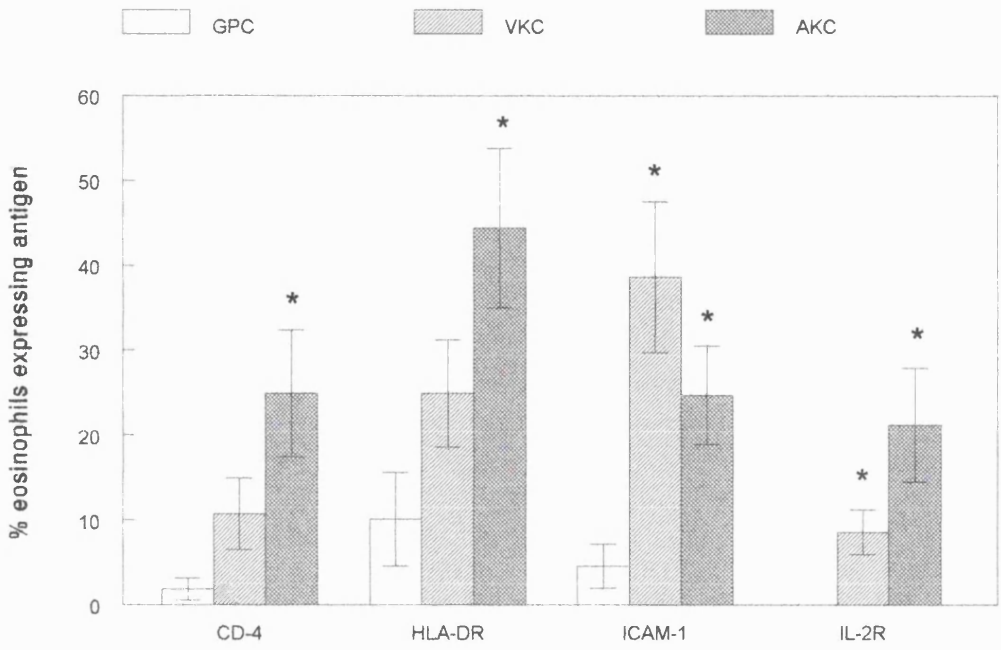


Figure 5.4. Conjunctival eosinophil expression of CD4, HLA-DR, ICAM-1 and IL-2R by IHC. Mean (SEM) percentage of eosinophils expressing antigens (* = significant interdisease difference).

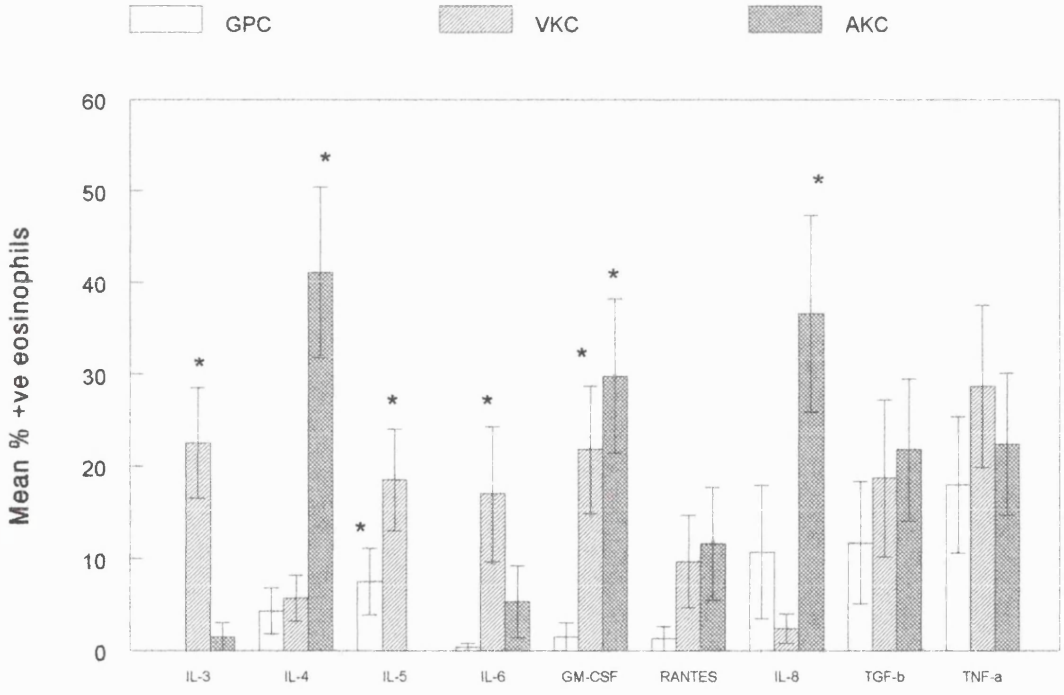


Figure 5.5. Conjunctival eosinophil expression of cytokines by IHC. Mean (SEM) percentage eosinophils staining for cytokine
(* = significant interdisease difference).

Table 5.1. Primary monoclonal antibodies used in eosinophil studies

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

Table 5.2: Leukocyte cell counts in the upper tarsal conjunctiva in chronic allergic eye diseases and controls

	Normal	GPC	VKC	AKC
Eosinophils	0.1 ± 0.06	21.6 ± 8.2 *p=0.0002*	16.2 ± 4.3 *p=0.0001*	3.6 ± 0.9 *p=0.0003*
Neutrophils	2.9 ± 0.4	31.0 ± 10.5 *p=0.001*	20.5 ± 7.3 *p=0.0002*	70.1 ± 13.6 *p=0.0002*
$CD4^+$ cells	3.3 ± 0.5	16.8 ± 4.3 *p=0.0006*	18.1 ± 4.4 *p=0.0002*	11.7± 2.6 *p=0.02*
$CD8^+$ cells	5.5 ± 1.0	25.5 ± 8.9 *p=0.003*	14.7 ± 2.8 *p=0.01*	6.2 ± 1.6
CD4/CD8	0.7 ± 0.1	0.8 ± 0.1	1.6 ± 0.4	2.4 ± 0.8 *p=0.01*
B cells	6.6 ± 1.1	39.8 ± 10.5 *p=0.002*	31.1 ± 5.7 *p=0.0002*	15.2 ± 1.9 *p=0.002*
Plasma cells	1.2 ± 0.5	9.3 ± 2.4 *p=0.005*	7.4 ± 0.8 *p=0.003*	5.7 ± 1.0 *p=0.001*
Macrophages	3.8 ± 0.5	7.5 ± 1.3 *p=0.02*	13.7 ± 2.5 *p=0.0008*	10.2± 1.3 *p=0.0005*
Mast cells	1.3 ± 0.3	15.7 ± 4.6 *p=0.0002*	8.2 ± 1.6 *p=0.0002*	8.6 ± 2.1 *p=0.0002*
Total leukocytes	25.9 ± 1.8	169.4 ± 46.5 *p=0.002*	129.9 ± 12.6 *p=0.002*	127.1 ± 2.1 *p=0.002*

Results expressed as mean ± SE cell count/mm^2 substantia propria. p values shown for significant differences in cell counts between allergic disease and normal.

Chapter 6

The role of conjunctival epithelial cells in chronic ocular allergic disease

6.1 Introduction

6.1.1 The role of conjunctival epithelial cells in ocular defence and immunology

The conjunctival epithelium is 2 to 5 cell layers thick, has a complex arrangement of microvilli and microplicae and contains goblet cells, Langerhans cells, some leukocytes and melanocytes (Records, 1988; Bron et al., 1985). Cells are joined by desmosomes and the surface cells by tight junctions (Chandler and Gillette, 1983). It has long been thought that the most important role of the conjunctival epithelial cell layer in ocular surface defence and inflammation is its barrier function together with its importance in stabilising the tear film (Chandler and Gillette, 1983; Smolin, 1985). However, recent evidence suggests that epithelial cells of the conjunctiva and of non-ocular mucosae may play an active role via expression of adhesion molecules, MHC class II molecules and other surface antigens, and via the release of pro-inflammatory lipid and cytokine mediators (Devalia and Davies, 1993; Jones et al., 1994; Raeburn and Webber, 1994; Campbell et al., 1994; Levine, 1995; Stadnyk, 1994).

6.1.2 Objectives

The aim of the study was to investigate whether such an active role is played by conjunctival epithelial cells in chronic ocular allergic inflammation and, if so, whether this role is different in disorders with corneal involvement. The aims of this study were, firstly, to perform a descriptive study to examine the conjunctival epithelial cell expression of immunoreactive pro-allergic cytokines, MHC class II molecules and ICAM-1 in patients with GPC, VKC and AKC. Although the presence and distribution of substantia propria immunocytological markers and cytokines in these disorders have been described (Maggi et al., 1991; Foster et al., 1991; Metz et al., 1996, Montan et al., 1995) very little is known about the expression of such molecules on conjunctival epithelial cells. Secondly, we aimed to investigate the hypothesis that epithelial cell function, measured by expression of cytokine, MHC class II molecules and ICAM-1, is different in the disorders which affect the cornea (AKC and VKC) compared with the disorders that do not (GPC), paralleling similar differences shown in the function of T cells and eosinophils (Chapters 5 and 6). The choice of cytokines investigated was dictated by their relevance to allergic inflammation and whether there was any published precedent for epithelial cell production.

6.2 Methods

6.2.1 Subjects and biopsies

Conjunctival biopsy specimens from the central superior tarsal conjunctiva were obtained from subjects with VKC (n=10, 2 female, age 20.0 ±1.6 {12-29] years), GPC (n=10, 3 female, 41.8 ±6.3 [20-73] years) and AKC (n=10, 3 female, age 27.9 ±2.3

[21-46] years). All subjects with VKC or AKC had current or previous corneal involvement. Specimens were obtained from 10 normal, non-age matched, non-atopic control subjects (6 female, mean age 55.2 ±7.3 [19-85] years). Specimens were embedded in GMA resin.

6.2.2 Immunohistochemistry

One-colour IHC was performed on 2μm sections using mouse anti-human monoclonal antibodies (Table 6.1) and AEC as chromagen. Appropriate positive and negative controls were performed including the use of an anti-cytokeratin monoclonal antibody (epithelial marker) as the primary antibody for a positive control.

6.2.3 Cell counts and statistical analysis

Epithelial cells staining positively were identified by a red deposition of AEC reaction product (Fig. 6.1). As the epithelial cell layer thickness is variably increased in chronic allergic eye disease (Morgan, 1971; Allansmith et al., 1981b) and because there was frequent confluence of staining, it was deemed not accurate to simply count the numbers of positively stained epithelial cells per high-power microscopic field. For each section, positive immunostaining of epithelial cells was graded in a masked fashion using a four-point scale: 0 (absent), 1 (positivity on ≤ 25% of epithelial cells), 2 (positivity on 25 to 50% cells), 3 (positivity on 50 to 75% cells), 4 (positivity on 75 to 100% epithelial cells) (Ciprandi et al., 1993a). Results were expressed as the number of samples containing positively stained epithelial cells and as the mean staining grade.

To analyse the differences between the disease groups, Fisher's exact test was used to compare the numbers of samples containing positive epithelial cell staining (two-sided p values given) and the Mann-Whitney U test (two-tailed) was employed to compare staining grades.

6.3 Results

6.3.1 ICAM-1 and HLA-DR

ICAM-1 and HLA-DR were not expressed on normal conjunctival epithelial cells, although some dendritic cells positive for HLA-DR were present (Figure 6.1b). There was conjunctival epithelial cell expression of both ICAM-1 and HLA-DR in the diseased tissue (Fig. 6.2). HLA-DR was expressed on 6/10 of GPC samples (staining grade 1.1±0.3, $p=0.005$), on 9/10 of VKC samples ($p=0.005$; staining grade 2.3±0.3, $p<0.001$) and on 10/10 AKC samples ($p=0.001$; staining grade 2.4±0.3, $p<0.001$). ICAM-1 was expressed on 6/10 GPC samples (staining grade 0.8±0.2, $p>0.05$), on 10/10 VKC samples ($p=0.001$; staining grade 2.0±0.2, $p<0.001$) and on 9/10 AKC

samples (p=0.02; staining grade 2.0±0.4, p=0.003). Epithelial cell expression of both HLA-DR (AKC p=0.02, VKC p=0.02) and ICAM-1 (AKC p=0.03, VKC p=0.008) was greater in AKC and in VKC than in GPC.

6.3.2 Cytokines which localised to normal conjunctival epithelial cells

Interleukin (IL)-8 (3/10 samples, staining grade 0.4±0.23), RANTES (1/10 samples, staining grade 0.1±0.1), IL-6 (1/10 samples, staining grade 0.1±0.1) and TNF-α (3/10 samples, staining grade 0.4±0.2) all localised to epithelial cells in normal conjunctiva (Fig. 6.3). There were no statistically significant differences in the expression of IL-6 and TNF-α between normal and allergic epithelial cells (IL-6 expression was raised in the allergic disorders, but did not reach significance) nor were there any inter-disease differences. There was increased expression of RANTES in all the allergic disorders compared to normal subjects: in GPC (6/10, p=0.04; 1.0±0.4, p=0.04), AKC (6/10, p=0.04; 0.8 ±0.2, p=0.02) and VKC (7/10, p=0.02; 1.4±0.3, p=0.005). There was increased expression of IL-8 in GPC (7/10 samples, staining grade 1.6±0.4) compared with normal subjects (p=0.04) and compared with VKC subjects (2/10 samples, staining grade 0.3±0.2, p=0.04) and AKC subjects (3/10 samples, staining grade 0.4± 0.2, p=0.04).

6.3.3 Cytokines localising only to epithelial cells in allergic disorders

Normal conjunctival epithelial cells did not express GM-CSF and IL-3. GM-CSF was expressed by epithelial cells in all the disorders. There was increased epithelial cell expression of GM-CSF in AKC (7/10, 1.5 ±0.4) compared with GPC (1/10 p=0.02; 0.2±0.2 p=0.01) and VKC (2/10; 0.3±0.2 p=0.02). IL-3 was expressed in VKC (1/10, 0.2±0.2) and AKC (2/10, 0.2±0.1) to equal degrees, but not in GPC.

6.4 Discussion

6.4.1 ICAM-1 on conjunctival epithelial cells

Nasal and bronchial epithelial cells may express ICAM-1 and increased expression is seen in allergic disease which correlates with clinical severity (Vignola et al., 1993; Gosset et al., 1995; Montefort et al., 1992). ICAM-1 is not seen on normal conjunctival epithelial cells but is induced in inflammatory ocular surface conditions such as Sjogren's syndrome (Jones et al., 1994; Vorkauf et al., 1993). Corneal epithelial cells can express ICAM-1 and this is upregulated by IL-1 or in corneal inflammation (Brevdik et al., 1995; Goldberg et al., 1994). ICAM-1 has also been detected on conjunctival epithelial cells in VKC and after allergen challenge in out-of-season SAC (Tabbara et al., 1996; Ciprandi et al., 1993a). The results of this study show that conjunctival epithelial cells express increased surface ICAM-1 in chronic

allergic eye disease in which corneal disease is a feature. The ICAM-1/LFA-1 interaction is thought to play a principal role in the migration of inflammatory cells from the vascular compartment to sites of inflammation (Smith, 1993). Its presence on epithelial cells will allow direct interaction of the epithelial cells with leukocytes, possibly contributing to recruitment and retention of inflammatory cells and concentrating leukocytes and inflammatory damage around the epithelium.

6.4.2 HLA-DR on conjunctival epithelial cells

Human airway epithelial cells may express HLA-DR and bronchial and nasal epithelial cell HLA-DR expression is increased in asthma and nasal polyps (Vignola et al., 1993; Stoop et al., 1989). HLA-DR expression is induced on conjunctival epithelial cells in inflammatory conditions (Jones et al., 1994; Badouin et al., 1992) and this study has demonstrated a small increase in expression in GPC with markedly increased expression in chronic ocular allergic disease which affects the cornea (AKC and VKC). The presence of this molecule on epithelial cells raises the question of whether the cells have the ability to process and present antigen. Intestinal epithelial cells expressing class II MHC molecules can present antigen *in vitro* with preferential stimulation of $CD8^+$ T suppressor cells (Brandtzaeg et al., 1989; Bland and Warren, 1986). Whether HLA-DR^+ epithelial cells from the airway or conjunctiva are able to present antigen, and perhaps influence the proliferation of specific T cell subtypes, is at present unknown.

6.4.3 Conjunctival epithelial cell cytokines

Previous studies have shown that epithelial cells from a number of tissues, including the airway epithelium, can synthesise potent pro-inflammatory cytokines including IL-1, IL-3, IL-6, IL-8, GM-CSF, RANTES and TNF-α, MCP-1, GRO-α and GRO-γ (Raeburn and Webber, 1994; Levine, 1995; Stadnyk, 1994). Cytokine production is upregulated in airway epithelial cells in asthma and allergic rhinitis (Raeburn and Webber, 1994; Levine, 1995; Devalia and Davies, 1993) and may be reduced by anti-allergic therapy (Kwon et al., 1995; Davies et al., 1995; Devalia et al., 1995.

Normal corneal epithelial cells express cytokines such as IL-1β, IL-8, TGF-α and -β, PDGF-β, b-FGF and LIF (Li and Tseng, 1995) and an IL-3-like factor (Grabner et al., 1985). A conjunctival cell line, "Chang", produces IL-1-like and IL-3-like factors (Grabner et al., 1985; Schreiner et al., 1985) and PDGF, bFGF, IL-2, IFN-γ and TNF-α proteins have been detected in normal and pemphigoid conjunctival epithelial cells (Bernauer et al., 1993b). Normal conjunctival epithelial cells contain mRNA for IL-1, IL-6 and IL-8 and there is increased expression of IL-6 mRNA in Sjogren's syndrome

(Jones et al., 1994). The results presented here confirm the presence of IL-6, IL-8, and TNF-α in normal conjunctival epithelial cells and show that RANTES can be added to the list of cytokines found in normal conjunctival epithelial cells. It also appears that there is upregulation of certain cytokines (RANTES in all chronic ocular allergic disorders, IL-8 in GPC only), and that production of GM-CSF (in all disorders, but especially AKC) and IL-3 (VKC and AKC) is induced.

6.4.4 Role of conjunctival epithelial cell cytokines in ocular allergy

The epithelial cytokines which are upregulated or induced in chronic ocular allergic disease are typical of allergic inflammation and atopic disease (Bittleman and Casale, 1994). IL-3 and GM-CSF promote eosinophil inflammation typical of allergic diseases by their effects on eosinophil migration, survival and activation (Nicola, 1994). There is good evidence that these Th2-type cytokines are involved in the pathogenesis of other allergic diseases such as asthma, AD and nasal allergy (Robinson et al., 1992; Leiferman, 1994; Durham et al., 1992). Since the eosinophil is strongly implicated in the development of allergic corneal damage (see Chapter 6), it is of interest that conjunctival epithelial cell expression of IL-3 and GM-CSF, which promote eosinophil activity, was particularly increased in the disorders which involve the cornea. RANTES is one of the most potent eosinophil chemoattractants and has chemotactic effects on other cells (T cells, macrophages) involved in allergic inflammation (Baggiolini et al., 1994) and IL-8 is chemotactic for neutrophils and eosinophils (Erger and Casale, 1995). Both of these cytokines have been implicated in the pathophysiology of allergic mucosal disease (Schwiebert et al., 1996).

6.4.5 Conclusions

In addition to T cells, it is now recognised that mast cells, eosinophils and epithelial cells are significant sources of cytokines in non-ocular allergic inflammation (Devalia and Davies, 1993; Bradding et al., 1993; Moqbel et al., 1994). The results presented here show that epithelial cell cytokines are also present in the conjunctiva in chronic ocular allergic diseases and, as occurs in conjunctival T cells and eosinophils, there are different cytokine profiles in the epithelial cells in the different clinical disorders. The relative importance of these cytokine sources and their relationship to each other has not yet been determined. It may be that, as in non-ocular allergy, the actions of anti-allergic drugs such as nedocromil sodium and steroids are, at least in part, mediated by inhibition of epithelial cell cytokine synthesis (Davies et al., 1995; Devalia et al., 1995) The differences in the patterns of epithelial cell cytokines also opens the possibility for future development of more specific therapy in ocular allergy.

Chapter 6: Tables and figures

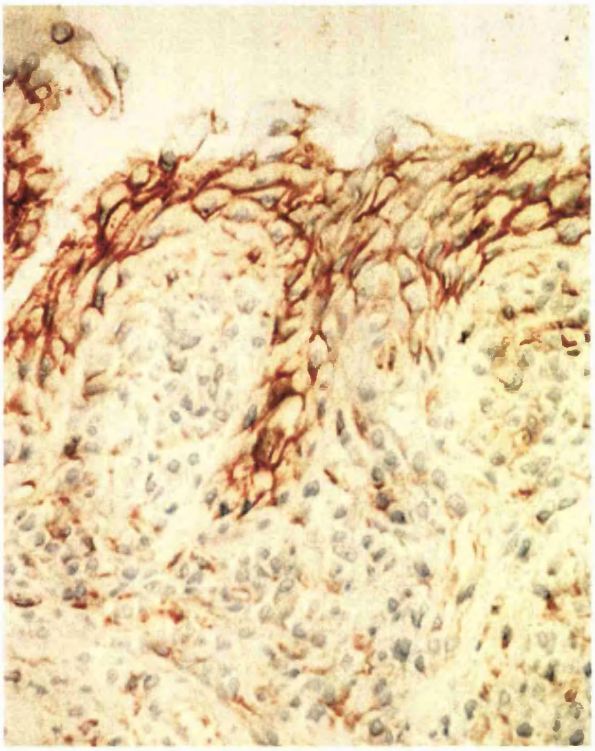


Figure 6.1a. Moderate expression of ICAM-1 on conjunctival epithelial cells in VKC giant papillae (x100, AEC).

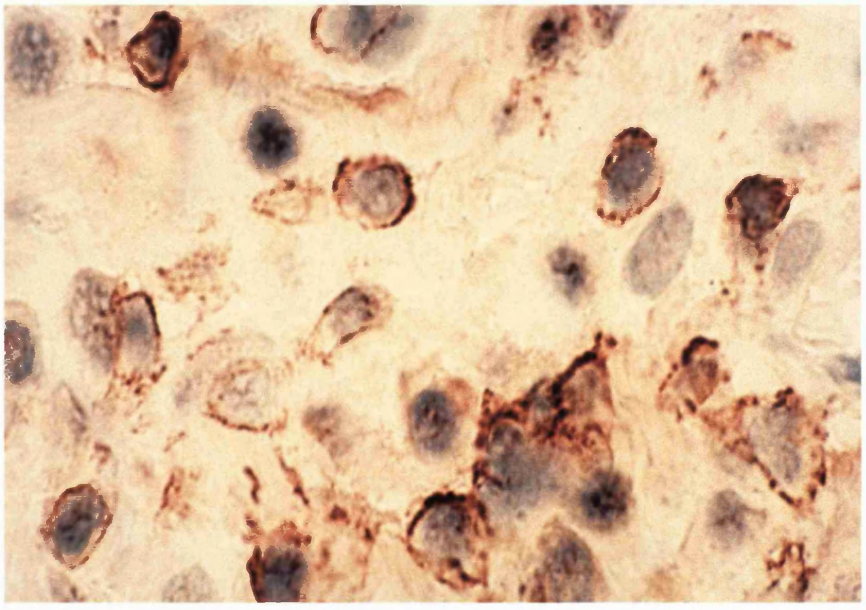


Figure 6.1b. Higher power view of expression of GM-CSF on conjunctival epithelial cells in AKC (x400, AEC).

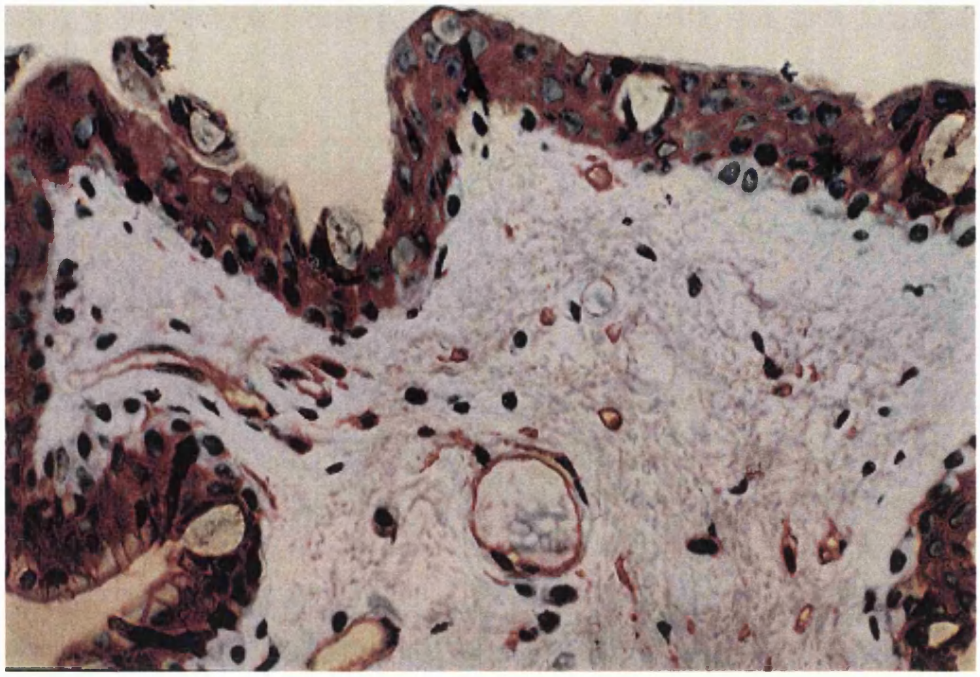


Figure 6.1c. Positive control slide showing very intense staining of conjunctival epithelial cells for cytokeratin using AE-1 mab (x200, AEC).

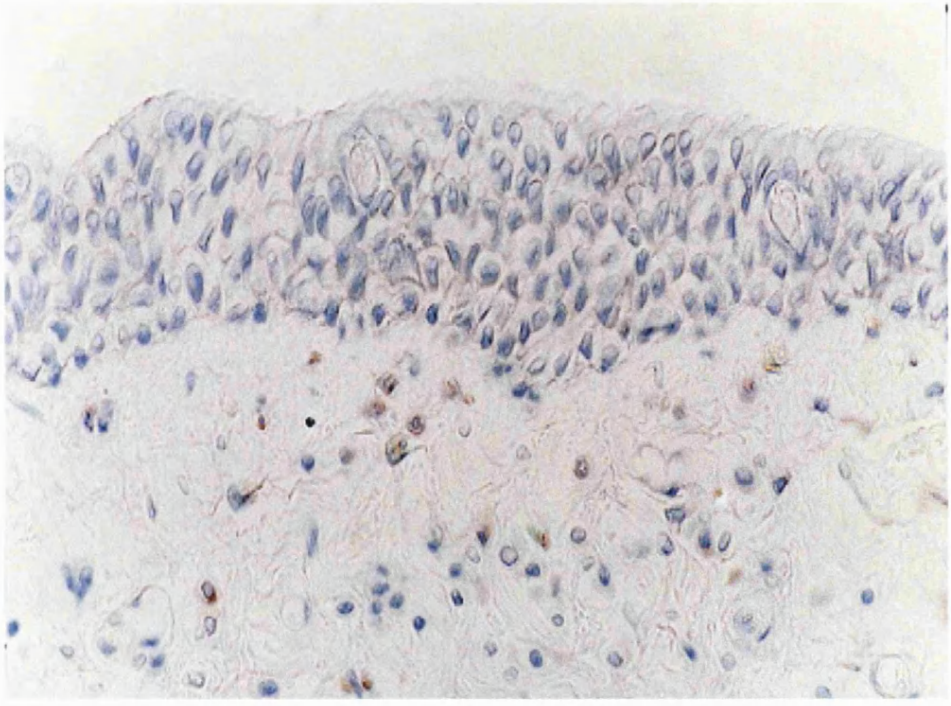


Figure 6.1d. Negative control slide showing no staining of conjunctival epithelial cells using an irrelevant antibody (x200, AEC).

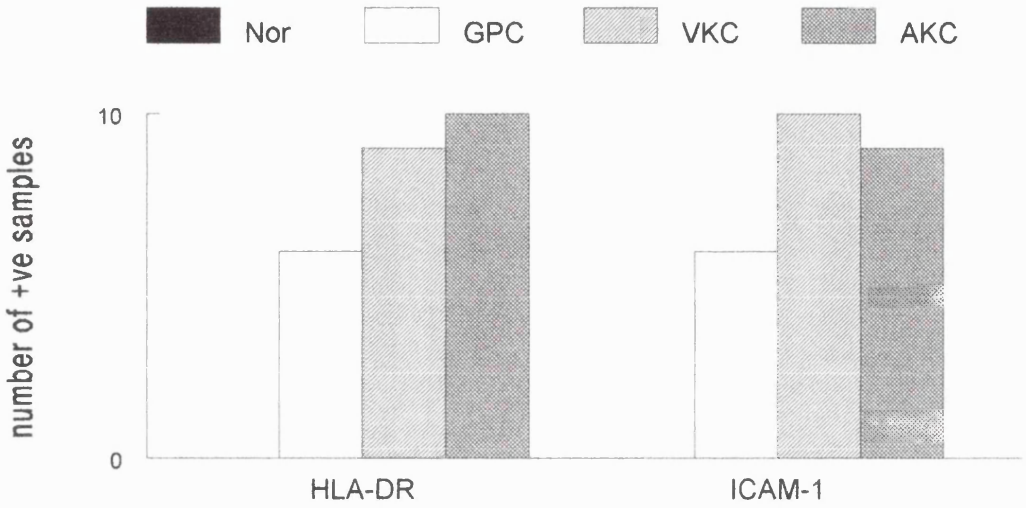


Figure 6.2a. Number of biopsies with epithelial cell expression of HLA-DR and ICAM-1.

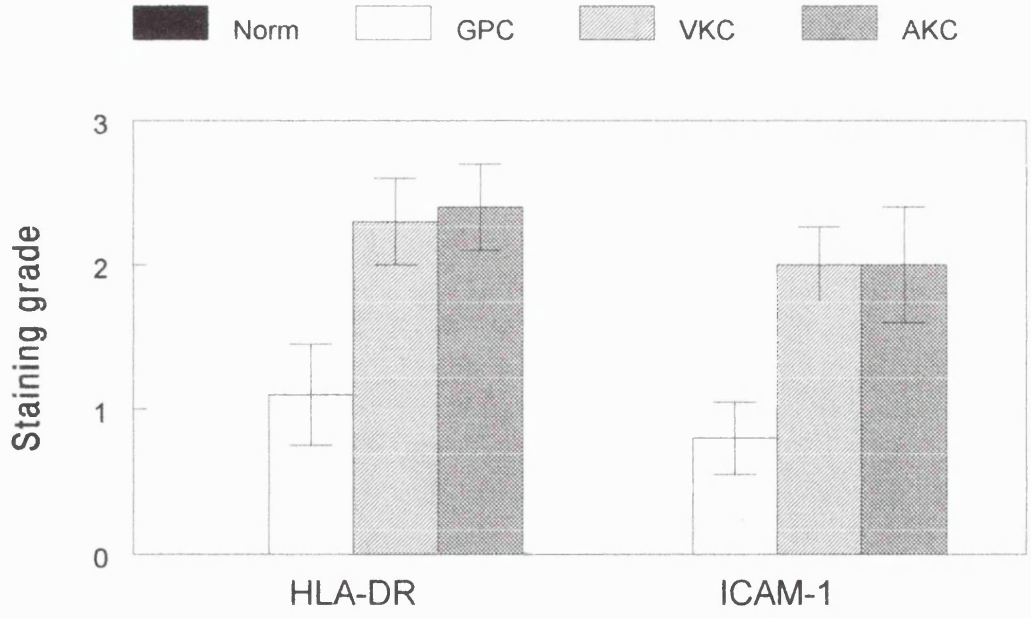


Figure 6.2b. Mean (SEM) epithelial cell staining grade for HLA-DR and ICAM-1.

Figure 6.2. Conjunctival epithelial cell expression of HLA-DR and ICAM-1 by IHC.

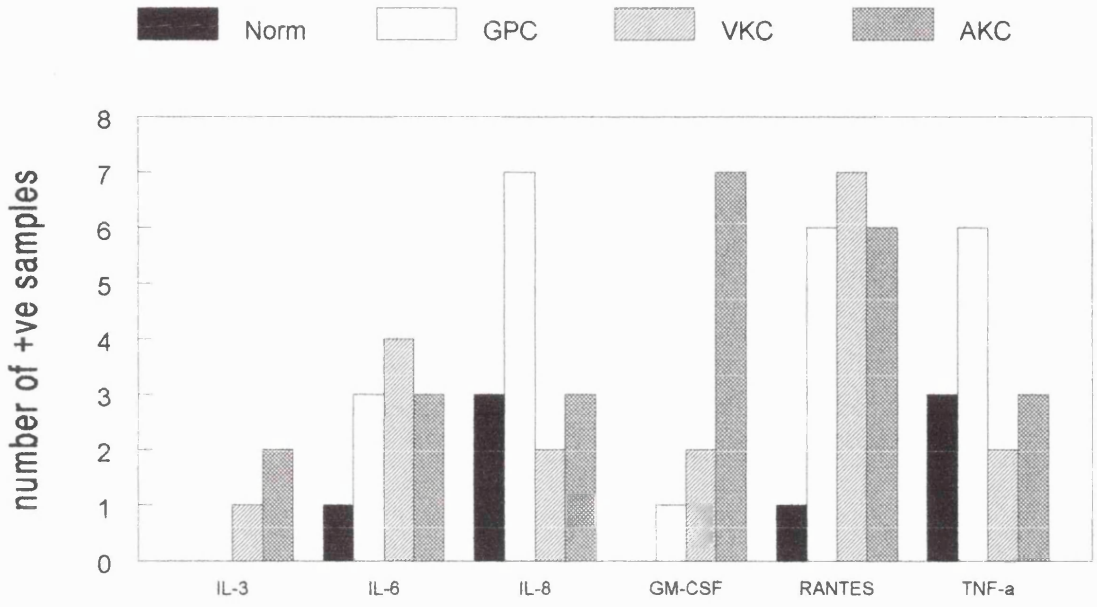


Figure 6.3a. Number of biopsies with epithelial cell expression of cytokines.

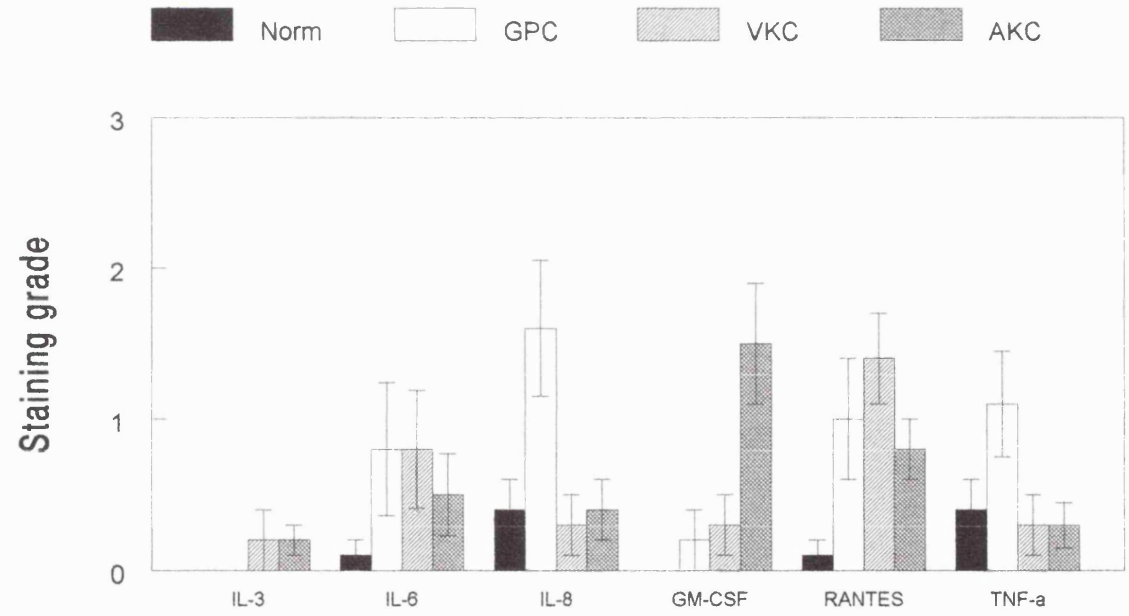


Figure 6.3b. Mean (SEM) epithelial cell staining grade for cytokines

Figure 6.3. Conjunctival epithelial cell expression of cytokines by IHC.

Table 6.1: Primary monoclonal antibodies used in studies of epithelial cells

Monoclonal antibody	Specificity	Source
HLA-DR	MHC class II molecules	Dako
CD-54	ICAM-1, intercellular adhesion molecule-1	Dako
AE-1	Anti-cytokeratin antibody	ICN Pharmaceuticals
IL-3	Interleukin-3	Genzyme
IL-6	Interleukin-6	Genzyme
GM-CSF	Granulocyte-monocyte colony stimulating factor	Genzyme
RANTES	Regulated upon Activation, normal T cells expressed and secreted	R&D Systems
IL-8	Interleukin-8	Genzyme
TNF-α	Tumour necrosis factor-α	Genzyme

Chapter 7

A randomised, placebo-controlled trial of topical cyclosporin A in steroid-dependent atopic keratoconjunctivitis

7.1 Introduction

7.1.1 AKC: a different disease from VKC

AKC and VKC are serious ocular allergic diseases and can cause visual loss through corneal involvement and conjunctival cicatrisation (Colby and Dohlman, 1996; Akova et al., 1994). Unlike VKC, which usually resolves spontaneously (Buckley, 1989), AKC persists for many years, with a high rate of significant visual impairment (Foster and Calonge, 1990). Both groups are often heavily reliant on topical steroids (Hingorani and Lightman, 1995) which, via their many serious side-effects (Jaanus, 1989), can contribute significantly to visual reduction in these patients. $CD4^+$ T lymphocytes are the predominant infiltrating cell type in AKC and VKC (Metz et al., 1996) and, although the pathogenesis is not fully understood, T cell-mediated inflammation appears to be a key feature. The T cell subsets, as defined by their cytokine co-localisation, present in AKC have been shown to be different to those in VKC (Chapter 4).

7.1.2 Actions of cyclosporin A in ocular allergy

CsA, which inhibits Th lymphocytes, has been used successfully in topical form (2% dissolved in oil) to treat VKC, with an improvement in symptoms and clinical signs and a dramatic effect as a steroid-sparing agent (Secchi et al., 1990a & 1990b; Bleik and Tabbara, 1991; Ben Ezra et al., 1986, 1988 & 1993). The role of CsA in the treatment of allergy is likely to relate not only to its inhibition of T cell-dependent mechanisms but also to its inhibitory effects on eosinophil activation, mediator and cytokine release (Borel et al., 1996).

7.1.3 Objectives

The effect of topical CsA in AKC might be different from that in VKC, given the differences in T cell subsets involved, although oral CsA has been used with some success in a small open trial (Hoang-Xuan et al., 1997). The aim of this trial was to investigate the hypothesis that topical CsA acts as a steroid-sparing agent in AKC and also produces clinical benefit.

7.2 Methods

7.2.1 Patients and randomisation

Twenty-one patients (9 female, age 34.7 ±2.9 [18-66] years) with steroid-dependent AKC were recruited. Patients were randomly assigned to receive either CsA 2% in maize oil or placebo (vehicle) eye drops four times daily to both eyes, in addition to their usual treatment, for three months. Due to its highly lipophilic nature, CsA must be dissolved in oil to achieve a concentration of 2% for topical ocular use. Both

patients and clinicians were masked to the identity of the drops used and drops were identical in appearance and in identical bottles. To provide evidence for successful masking, both patients and clinicians were asked to try to identify whether the drops used were active drug or placebo at the end of the trial. If masking was 100% successful, it would be expected that no more than 50% of assessments of treatment status (made either by clinicians or patients themselves) would be correct. Among the 21 patients, 13 of their assessments were correct and 8 were wrong ($\chi^2_{1df} = 0.86$, $p>0.05$); for the clinicians, 14 of their assessments were right and 7 were wrong ($\chi^2_{1df} = 1.5$, $p>0.05$), suggesting that both patients and clinicians were successfully masked to treatment identity.

7.2.2 Clinical monitoring

Patients were identified for trial suitability and willingness to participate over 1 year preceding the trial and both groups were assessed simultaneously with the onset of the trial staggered evenly in both groups over 5 months to allow time for the same physician to assess the patients. Patients underwent a full medical and ophthalmic history and ophthalmic examination before the onset of the trial drops and were reviewed at 1 week and then monthly. Symptoms of itch, soreness (burning, discomfort), redness, tearing, discharge, blurring of vision and photophobia were recorded at each visit and graded 0 (none), 1 (mild), 2 (moderate), 3 (marked) or 4 (severe) by the clinician on questioning. The patients also kept a daily diary of symptoms (using the same grading) and of medication usage. Clinical signs were graded by the physicians for the lids (ptosis, lid skin dermatitis, lid margin hyperaemia, meibomian gland abnormality, lid margin thickening and rounding, lid margin anatomical distortion), for the conjunctiva (hyperaemia and oedema for the bulbar conjunctiva, hyperaemia, infiltration and papillae for the inferior tarsal conjunctiva and for the superior tarsal conjunctiva, subepithelial conjunctival scarring, cicatrisation [i.e. forniceal foreshortening, symblepharon, obliteration of the canthal architecture], limbitis) and for the cornea (tear film deficiency, epithelial disease, opacity, stromal thinning, neovascularisation, lipid deposition). These were all graded from 0 to 4 in the same way as the symptoms except for meibomian gland dysfunction (0= orifices not dilated, fluid expressible and normal, 1 = dilated or abnormal orifices, fluid expressible and normal, 2 = dilated or abnormal orifices, fluid expressible but abnormal, 3 = dilated or abnormal orifices, fluid not expressible), ptosis (0= none, 1 = upper lid covers 2-4mm cornea, 2 = lid covers 4-6mm, 3 = covers 6-10mm, 4 = covers >10mm), papillae (Fig. 7.1), corneal epithelial disease (Fig. 7.2). Totals were obtained for lids, conjunctiva and cornea and then a sum total score also derived. A record of

disease complications (e.g. plaque ulcer) and steroid complications (e.g. raised intraocular pressure) which developed during the trial was also kept.

7.2.3 Topical steroid use: reduction and monitoring

The clinical response was used to reduce and stop topical steroids, when possible, and steroid drop usage per week was scored, with allowances made for the relative potency of the different topical steroid preparations by using a multiplication factor (i.e. number of drops per week multiplied by five for prednisolone acetate 1%, by four for dexamethasone 0.1%, by three for fluoromethalone 0.1%, by two for prednisolone phosphate 0.3% and by one for prednisolone phosphate 0.1%) (Jaanus, 1989). Patient compliance with drop therapy was assessed from the patients' diary cards and by examination of the remaining volume of drops in the bottles. Clinicians noted and graded trial drop side-effects and patients kept a note of side-effects of the trial drops on their diary cards. At the end of the trial, both patients and clinicians were asked to give an overall rating for the trial drops as poor, moderate, good or excellent, based on improvement in symptoms and signs, drop tolerance and ability to reduce or stop concurrent steroid drop treatment.

7.2.4 Statistical analysis

For statistical analysis of data from the two groups (which were analysed according to their original group assignation), continuous variables were compared using the Mann-Whitney U tests and dichotomous data using the Fisher's exact test (2-tailed).

7.3 Results

7.3.1 Age and gender

All patients completed the full three month follow-up and there were no deviations from the planned protocol. Of the 21 patients in the trial, 12 received CsA (4 females, mean age ±SEM 33.9 ±3.9 years) and 9 received placebo (5 females, age 35.7 ±4.6 years) and there were no significant differences between the ages and sexes of the two groups.

7.3.2 Steroid-sparing effect (Fig. 7.3)

Cs A drops had a greater steroid-sparing effect than placebo drops. Nine of the 12 CsA patients were able to cease steroid therapy, which was a significantly greater portion than in the placebo group, in which only 1 of the 9 patients discontinued steroids ($p=0.01$). There was a difference between the two groups in the initial weekly steroid drop usage, with a higher mean (±SEM) drop use in the CsA group (88.1± 15.2) than in the placebo group (41.2±7.1, $p=0.04$). However, the final steroid use in

the CsA group (2.6±1.4) was significantly lower than in the placebo group (27.7±17.7, p=0.005). The mean reduction in steroid drop usage was significantly greater in the CsA group (85.5±14.7) than in the placebo group (13.9±16.0, p=0.005).

The results also showed that it was possible to reduce the steroid dose in all trial patients except one (one patient in the placebo group was using more steroids at the end than at the beginning of the trial). In the CsA group, there was a highly significant reduction in the mean weekly steroid drop usage from 88.1 (±15.2) initially to 2.62 (± 1.4) at the end of the trial ($p<0.0001$) but in the placebo group there was also a significant reduction in steroid usage from 41.2 (±7.1) to 27.7 (±17.7) (p=0.02).

7.3.3 Clinical signs

There was no significant difference in the total clinical sign scores, nor in any of the individual scores, between the two groups at the beginning of the trial (Fig. 7.4). The final mean total clinical sign score was significantly less in the CsA group (13.3±1.8) than in the placebo group (25.2±2.9, p=0.004). Those patients receiving CsA had a significant reduction in total clinical sign score from 27.3±3.6 initially to 13.3±1.8 at the end of the trial (p=0.003) but in the patients receiving placebo there was no significant difference between the total sign score at the beginning (26.8±3.2) and at the end (25.2±2.9) of the trial. The mean improvement in total clinical sign score was significantly greater in the CsA group (14.0±3.4) than in the placebo group (1.5±1.9, p=0.02). Similar results were seen when clinical sign totals were analysed for the lids and particularly the conjunctiva. There was no significant change in mean conjunctival score for the placebo group (initial 17.2±2.3, final 17.2±2.1) but a highly significant change for the CsA group (initial 16.85±1.8, final 8.5±1.0, p=0.006) and there was a mean improvement in the conjunctival score for CsA patients (8.3±2.0) compared with a mean *worsening* of 0.06±1.5 for placebo patients (p=0.008).

7.3.4 Symptoms

There were no differences between the two groups for the initial or final symptoms scores as measured by patients and by clinicians. However, there were significant reductions in the mean symptom score for the CsA group as measured by patients (initial score 11.3±1.3, final score 6.2±2.0, p=0.005) and by clinicians (initial score 10.8±1.6, final score 5.4±1.3, p=0.02) but no significant change for the same scores in the placebo group (patients' initial score 10.3±1.6, final 8.5±2.0; clinician's initial score 9.5±2.2, final 9.0±1.6).

7.3.5 Side-effects

The most common side-effect was blurring of vision lasting from 30 seconds to 3 hours after drop instillation. This was present in nine placebo patients and five CsA patients ($p=0.01$). Visual blurring that was considered significant by patients was present in six placebo patients and five CsA patients. Some stinging upon drop instillation was experienced by 12 CsA patients and two placebo patients ($p=0.0006$) and intense stinging by nine CsA patients and one placebo patient ($p=0.01$). Other drop side effects, all occurring after drop instillation, were tearing (5 CsA, 2 placebo), increased discharge (1 CsA, 1 placebo), redness (0 CsA, 2 placebo) and puffiness of lids (1 CsA, 0 placebo). There were two cases with more serious reactions. One patient using CsA developed lid skin maceration which began just before the end of the trial. This resolved quickly on stopping the CsA and using topical steroid cream to the skin. Another patient using placebo developed a marked allergic reaction of the lid skin and conjunctiva, necessitating cessation of the drops for a few days at week four and permanent cessation of the trial drops at week 10. It was later discovered that the patient had a sensitivity to maize antigens which probably underlay this reaction. There were no episodes of CsA-related corneal epitheliopathy.

Because of the various side-effects of the drops, four placebo patient and five CsA patients were unable to tolerate the drops more than two to three times daily.

7.3.6 Estimation of overall effect (Fig. 7.5)

Despite the frequency of side-effects, eight of the 12 CsA patients (66.6%) rated the trial drops as good or excellent compared with three of the nine placebo patients (33.3%). The clinician also rated the trial drops as good or excellent in 11 of the 12 CsA patients (91.6%), compared with none of the placebo patients ($p<0.0001$).

7.4 Discussion

7.4.1 Safety of topical CsA is much greater than for topical steroids

Topical CsA has been used to treat a number of inflammatory ocular surface conditions (e.g. Sjogren's syndrome, ocular cicatricial pemphigoid) as well as VKC (Power et al., 1993; Holland et al., 1989 & 1993; Leigener et al., 1990; Zierhut et al., 1989; Belin et al., 1989). Its systemic absorption is minimal and therefore it does not cause any systemic side-effects (Secchi et al., 1990a & b; Bleik and Tabbara, 1991; Holland et al., 1993; Belin et al., 1989) In reports of prolonged topical CsA use the only serious side-effects are lid maceration (which developed in one patient in this trial) and corneal epitheliopathy, both of which resolve on cessation of treatment and which do not necessarily preclude further use of CsA (Secchi et al., 1990 a & b; Ben Ezra et al., 1986; Power et al., 1993; Belin et al., 1989) Topical CsA does not carry the sight-

threatening complications of topical steroids, such as glaucoma, cataract and potentiation of corneal infection.

7.4.2 Beneficial effect of topical CsA in steroid-dependent AKC

In this trial, topical CsA 2% allowed patients with steroid-dependent AKC to be weaned off topical steroids and, in addition, appeared to be an effective and safe means to reduce symptoms and signs, even whilst the steroid dose was lowered. Patients who received placebo did not have a statistically significant improvement in symptoms and signs, but were able to reduce the dosage of steroids used without a deleterious effect. This is partly because of the well-recognised, marked placebo-effect seen in allergic disease (both ocular and systemic) but may also owe something to more frequent review and encouragement by a clinician than usual and perhaps also to the lubricant effect of the maize oil vehicle, which many patients felt subjectively to have a beneficial effect. Although the measurements of clinical symptoms and signs and drop side-effects are subjective and therefore a possible source of imprecision, in clinical practice decisions are based upon just such judgements and therefore we deemed this acceptable. Although the ability of both patients and clinicians to guess which drop was administered (i.e. measurement of successful masking) did not reach statistical significance, both patients and clinicians were able to correctly identify the treatment more often than not. This therefore represents a possible source of bias.

7.4.3 Side-effects of topical CsA

In this trial, apart from one patient who had an allergic reaction to maize antigens, only one patient developed lid skin maceration and no patient developed drug-related corneal epitheliopathy, the two previously reported important side-effects of topical CsA. The lid skin maceration developed at the end of the trial period of 12 weeks (and resolved quickly with appropriate management). The number of patients developing such side-effects might therefore be greater were the period of therapy to be greater than three months, but in our experience (unpublished data) of using this drop for up to 18 months such complications are rare. However, drug-related symptoms of blurring (vehicle-related) which may last up to three hours and may prevent driving, and of intense stinging (CsA-related), sometimes causing severe blepharospasm for some time after instillation, make this drop difficult to tolerate and sometimes almost impossible to instil four times daily if the patient is working. In this group, despite these drop-related symptoms, the majority of patients rated CsA treatment overall as good or excellent. The patients in this trial were recruited from a tertiary referral centre, where patients may have unusually severe or steroid-dependent disease. Such high patient acceptance of CsA may not be repeated in a group of patients whose disease was less

severe or less steroid-dependent and may limit the use of CsA as a first line agent unless the patient is very well educated and motivated with regard to the risks of topical steroids.

7.4.4 Conclusion

The results of this trial suggest that topical CsA 2% is a safe and effective steroid-sparing agent in AKC and should be considered as an alternative to, or as an adjunctive therapy with, topical steroids.

Chapter 7: Figures

Superior tarsal conjunctiva
Grade for each zone and sum:
0 = no/minimal micropapillae
1 = micropapillae <0.3mm
2 = macropapillae 0.3-1mm
3 = giant papillae >1mm

Inferior tarsal conjunctiva
Grade, as one zone, as above

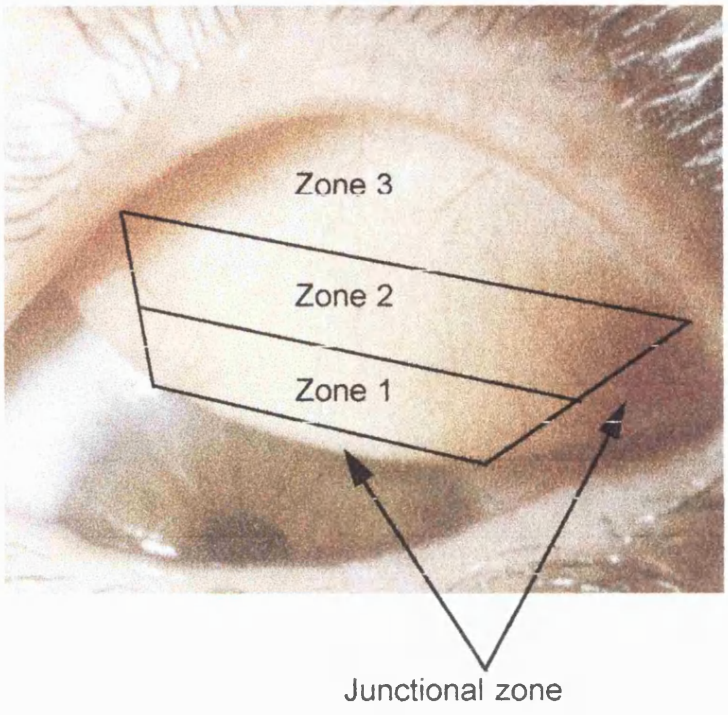


Figure 7.1. Grading of clinical sign score for papillae.

Score epitheliopathy for each third of cornea and sum

0 = none
1 = mild
2 = moderate
3 = severe
4 = very severe

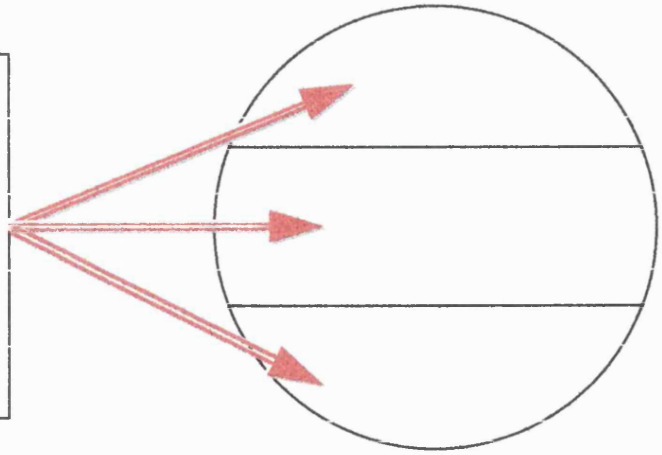


Figure 7.2. Grading of clinical sign score for corneal epitheliopathy.

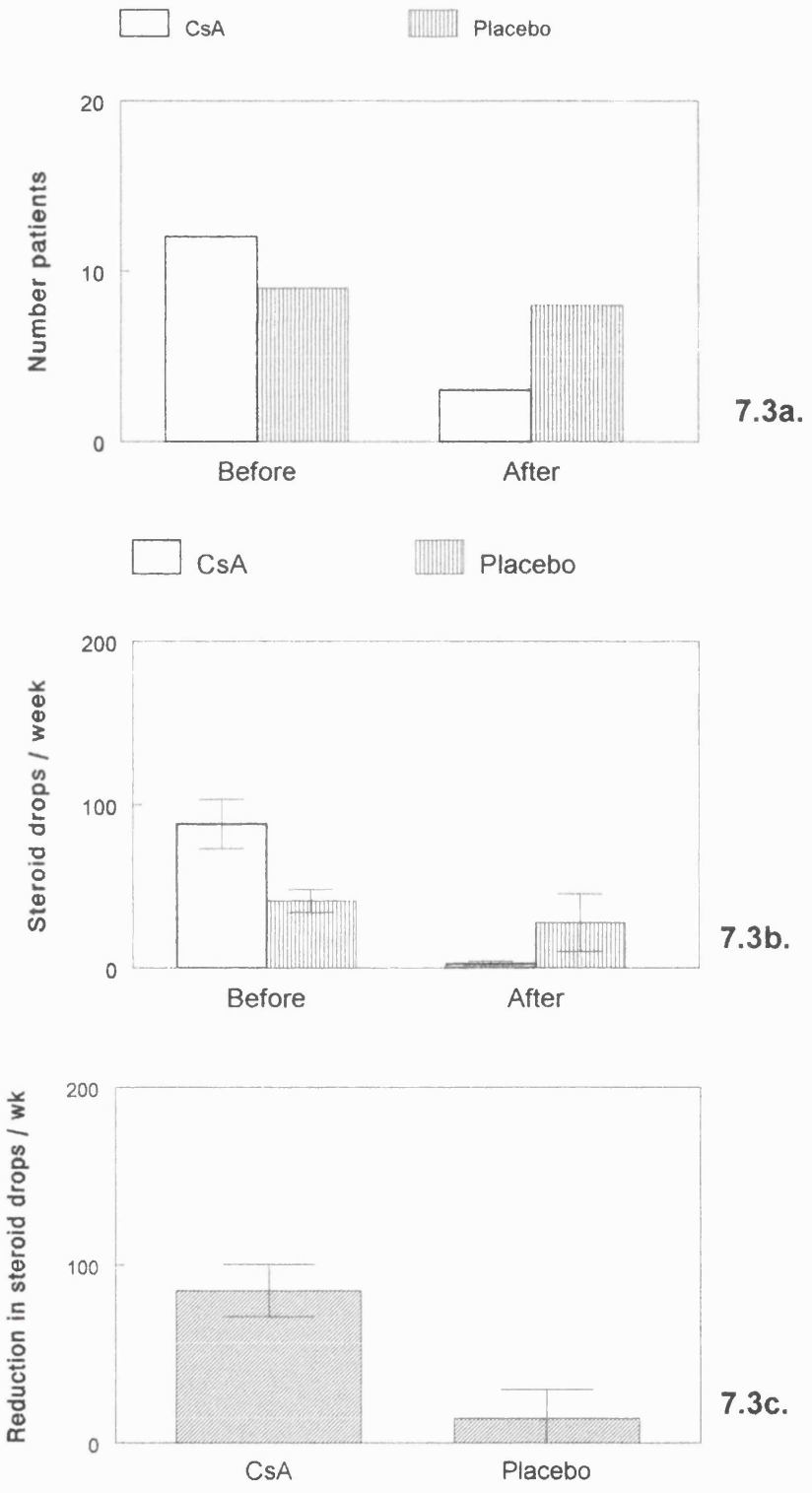


Figure 7.3. Changes in steroid drop use during trial of CsA. 7.3a. Number of patients requiring topical steroids before and after the trial; 7.3b. Mean (SEM) steroid drop use/week before and after the trial; 7.3c. Mean (SEM) reduction in steroid drop use/week over the trial period.

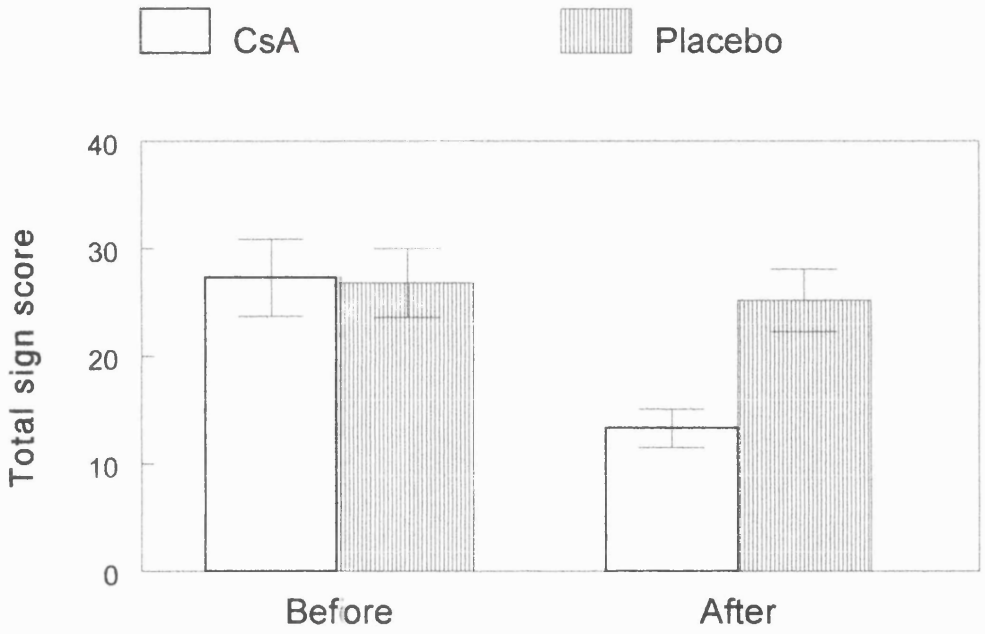


Figure 7.4a. Mean (SEM) total clinical sign score before and after the trial.

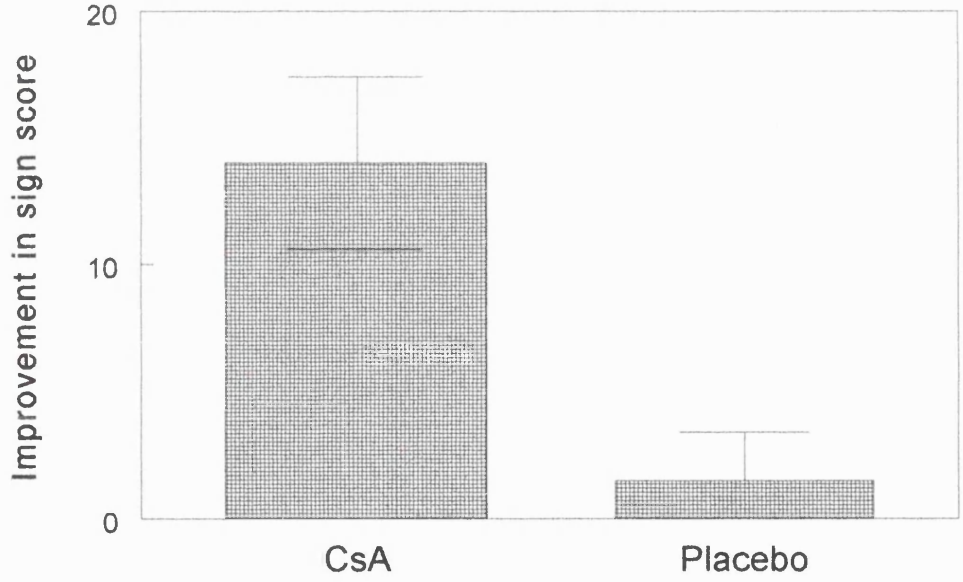


Figure 7.4b. Mean (SEM) decrease in clinical sign score before and after the trial.

Figure 7.4. Changes in total clinical sign score during trial of CsA.

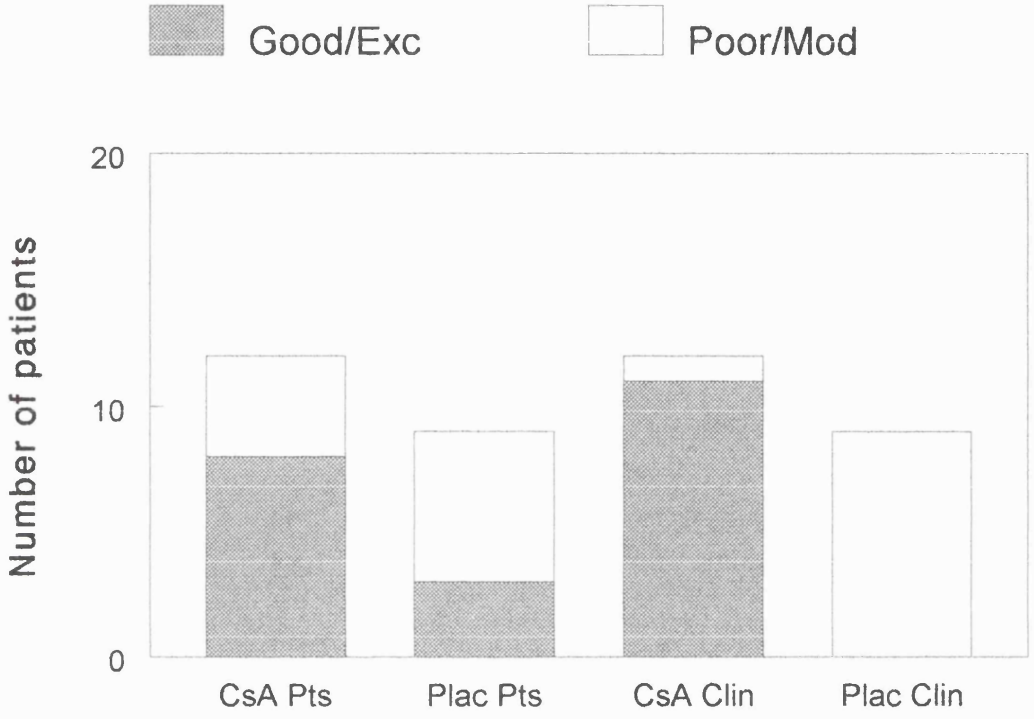


Figure 7.5. Overall rating by patients (Pts) and clinicians (Clin) of trial drops: number of subjects rated as good or excellent vs. number rated as poor or moderate.

Chapter 8

The immunomodulatory effects of topical cyclosporin A in atopic keratoconjunctivitis

8.1 Introduction

Atopic keratoconjunctivitis is the most severe form of ocular allergic disease, with a chronic course and a high prevalence of visual compromise (Foster and Calonge, 1990; Tuft et al., 1991; Akova et al., 1994). Patients with AKC are frequently treated with topical steroids, which have sight-threatening adverse effects (Foster and Calonge, 1990; Hingorani and Lightman, 1995; Jaanus, 1985). Topical CsA produces clinical improvement in VKC and acts as a steroid-sparing agent (Secchi et al., 1990 a & b; Bleik and Tabbara, 1991; Ben Ezra et al., 1986, 1988 & 1993). Topical CsA appears to have similar beneficial effects in AKC (Chapter 7).

T lymphocyte-mediated chronic inflammation appears to be important in the immunopathogenesis of both AKC and VKC (Bhan et al., 1992; El-Asrar et al., 1989a; Metz et al., 1996; Foster et al., 1991). There are functional differences in the T cells involved in the two disorders, with Th2-type cells predominant in VKC and a shift towards Th1-type cells in AKC (Chapter 4). A previous study showed immunomodulatory effects of topical CsA on VKC conjunctiva, with a reduction in inflammatory cell numbers, particularly T cells, HLA-DR$^+$ cells and plasma cells (El-Asrar et al., 1996). The aim of this study was to examine the immunomodulatory effects of topical CsA in conjunctival tissue from patients with AKC.

8.2 Methods

8.2.1 Subjects, randomisation of therapy and biopsies

Eight patients (2 female, age 33.2±4.8 [22-61] years) with AKC were recruited to the study. Use of all topical therapy apart from lubricants was ceased two weeks before the trial and patients were randomly assigned (by computer-generated randomisation) to receive CsA 2% in maize oil or placebo (vehicle) eye drops four time daily to both eyes for three months. Both patients and clinicians were masked to the identity of the drops and the drops were identical in appearance and in identical bottles.

Biopsies of the central superior tarsal conjunctiva were obtained from the subjects before starting treatment with CsA or placebo (but after 2 weeks without topical anti-allergic therapy) and another biopsy was taken at the end of the three months, while still using the trial drops. The biopsies were embedded in GMA.

8.2.2 Immunohistochemistry

IHC was performed on 2μm sections. One-colour IHC using appropriate primary antibodies (Table 8.1) was performed using AEC as chromagen for cell-counts and epithelial staining. For the localisation of cytokine to T cells, two-colour IHC was

performed using AEC to identify cytokine protein and DAB to identify CD3. Appropriate positive and negative controls were performed. H&E staining was performed and used to identify plasma cells.

8.2.3 Cell counts and statistical analysis

Cells staining positively for cell identification markers (e.g. CD20 for B cells) or for cytokine were identified by a red deposition of AEC (Figure 1). Plasma cells were identified on H&E sections. T cells staining for cytokine were identified by a brown or red-black combination colour of AEC and DAB (Figure 1).

Masked cell counts were performed to obtain mean counts per square mm of substantia propria. Individual leukocyte counts were summed for a total leukocyte count. For T cell double-staining, the results were expressed as the numbers per square mm of double-stained T cells. Epithelial cell staining was measured using a four-point scale and expressed as the mean staining grade.

A non-parametric statistical test (Mann-Whitney U test, two-tailed) was employed to analyse the data.

8.3 Results

8.3.1 Patient details and clinical changes

All patients completed the full trial and follow-up period. Four patients received CsA (all male, age 30.7±5.5 [22-47] years) and four patients received placebo (2 male, age 35.7±8.6 [22-61] years). Disease severity was graded (as described in Chapter 7) and initial grades for both symptoms and signs were similar in the treated and the control groups. Final grades for clinical signs were slightly better for the CsA group (mean ± SEM 13.0±0.9) than the placebo groups (24.4±3.5, p=0.06) and there was an improvement in clinical signs in the CsA group (mean ± SEM sign improvement 9.6± 4.3) compared with a worsening of clinical signs in the placebo group (-4.5±0.5, p=0.03).

8.3.2 Leukocyte numbers and epithelial staining

8.3.2.1 Pre-treatment

Before the onset of the treatment, the conjunctival infiltrate consisted of $CD4^+$ and $CD8^+$ T cells, $CD20^+$ B cells, plasma cells, eosinophils, neutrophils, mast cells and macrophages, with $CD3^+$ T cells the most frequently occurring cell type (mean ±SEM 5.2±3.9/mm^2) and the next most frequent types $CD20^+$ B cells (14.5±2.1/mm^2) and $CD68^+$ macrophages (12.8±1.5/mm^2). The CD4/CD8 ratio was 2.8±0.9. There were

no differences in the total cell count (CsA group 91.4±7.8/mm^2, placebo group 70.6± 20.2/mm^2) nor any of the counts of individual cell types between the placebo group and the CsA groups at this stage (Figure 2).

8.3.2.2 Post-CsA leukocyte numbers

After treatment with CsA, there was a significant reduction in the total leukocyte count from an initial count of 91.4±7.8/mm^2 to a final count of 41.5±3.5/mm^2 (p=0.03), but after treatment with placebo there was no significant change from the pre-treatment (70.6±20.2/mm^2) to the post-treatment (58.5±15.8/mm^2) total leukocyte count (Figure 2). After therapy with CsA, there was a significant reduction from initial to final values in the number of CD3$^+$ T cells (28.6±4.6/mm^2 to 9.3±1.6/mm^2, p=0.03), CD4$^+$ cells (from 18.2±2.5/mm^2 to 2.4±0.1/mm^2, p=0.03), CD8$^+$ cells (from 9.0± 1.4/mm^2 to 2.9±0.5/mm^2, p=0.03), CD20$^+$ B cells (from 17.5±1.1/mm^2 to 6.5± 1.2/mm^2, p=0.03), neutrophils (from 11.2±1.7/mm^2 to 3.1±0.8/mm^2, p=0.03) and CD68$^+$ macrophages (from 13.9±2.2/mm^2 to 3.9±0.8/mm^2, p=0.03). There were no significant differences between pre- and post-treatment values for the numbers of plasma cells, eosinophils and mast cells in the CsA-treated group. CD4/CD8 ratio reduced with CsA treatment from an initial value of 2.0±0.06/mm^2 to a final value of 0.8±0.1/mm^2 (p=0.03). Subject numbers were too small to obtain any useful information on a direct correlation between cell counts and clinical improvement.

8.3.2.3 Post-CsA HLA-DR, IL-2R and epithelial staining

There was a significant reduction in the number of HLA-DR$^+$ cells in the substantia propria (pre-treatment 24.2±2.1/mm^2, post-treatment 7.5±0.9/mm^2, p=0.03) after CsA (Figure 3) and the staining grade for HLA-DR positivity for the conjunctival epithelial cells was also reduced (pre-treatment 2.2±0.4/mm^2, post-treatment 0.7±0.2/mm^2) but this did not quite reach statistical significance (p=0.06) (Figure 4). There was a significant reduction in the number of IL-2R$^+$ (CD25$^+$) cells (pre-treatment 12± 1.1/mm^2, post-treatment 4.3±1.1/mm^2, p=0.03) in the substantia propria (Figure 3).

8.3.2.4 Post-placebo changes

There were no significant changes between pre-treatment and post-treatment values for any of the cell counts nor in the CD4/CD8 ratio and epithelial HLA-DR staining with placebo usage.

8.3.3 T cell cytokine expression

In the CsA group, the numbers of T cells staining for cytokines IL-3 and IL-5 were reduced but this did not reach statistical significance (IL-3 pre-treatment 1.7±0.6/mm^2,

post-treatment 0.8±0.4/mm^2; IL-5 pre-treatment 1.6±0.9/mm^2, post-treatment 0.6± 0.5/mm^2) and there was a statistically significant reduction in the numbers of T cells expressing IL-2 (pre-treatment 11.5±2.0/mm^2, post-treatment 4.2±0.3/mm^2) and expressing IFN-γ (pre-treatment 4.0±0.6/mm^2, post-treatment 0.1±0.08/mm^2) (Figure 5). There was no change in IL-4 expression in the CsA group and no significant change from initial to final values for any of the counts in the placebo group.

8.4 Discussion

8.4.1 Effects upon T cells

In vitro studies have shown that CsA blocks activation and proliferation of T lymphocytes, which arises in part from inhibition of the transcription of IL-2 (Kronke et al., 1984; Borel et al., 1996). CsA also inhibits the expression of IL-2R (Foxwell et al., 1990; Haczku et al., 1996). Our results show that this translates into a reduction in total T cell numbers in CsA-treated AKC conjunctiva, paralleling that previously published in CsA-treated VKC conjunctiva (El-Asrar et al., 1996), and the results also demonstrate a reduction in IL-2R expression and activation in conjunctival T cells in AKC. There was a reduction in the numbers of the T cell subsets CD4 and CD8, although the drop in the CD4/CD8 ratio shows that there was a relatively greater decrease in CD4 numbers. The CD4/CD8 ratio dropped to a mean below 1.0, which suggests a shift to a more physiological balance of T cell subsets, as the ratio is usually low in normal conjunctiva and raised in inflammatory disease states (Chan et al., 1988; Sacks et al., 1986a; Foster et al., 1991; Bernauer et al., 1993a; Bhan et al., 1982; Jakobiec et al., 1984).

8.4.2 Effects on HLA-DR expression

HLA-DR is a class II MHC antigen, a cell-surface glycoprotein involved in antigen presentation to, and activation of, T lymphocytes. Class II antigen expression occurs on leukocytes (e.g. Langerhans cells, macrophages, B cells) and may be induced on conjunctival epithelial cells in inflammatory conditions, including VKC and AKC (Chapter 6) although the functional significance of this is uncertain (Jones et al., 1994; Badouin et al., 1992). Our data shows a decrease in HLA-DR expression on cells in the substantia propria and also suggests a reduction of epithelial cell expression. CsA has been shown to reduce HLA-DR expression in macrophages and vascular endothelial cells (Buurman et al., 1986; Whistler et al., 1985; Groenewegen et al., 1985). This may be mediated by the ability of CsA to reduced IFN-γ transcription (Reem et al., 1983; Borel et al., 1996; Herold et al., 1986) as IFN-γ is a potent inducer of HLA-DR expression (including in ocular tissue) (Volk et al., 1985; El-Asrar et al., 1989b). Our data also demonstrate a large decrease in T cell expression of IFN-γ in

CsA-treated conjunctiva. CsA also appears to be able to directly inhibit the antigen-presenting function of APC such as Langerhans cells (Borel et al., 1996), which may further contribute to the observed reduction in T cell activation.

8.4.3 Effects on B cells, plasma cells and IgE

Both B cell proliferation and B cell antibody production have been shown to be inhibited by CsA *in vitro* (Lun et al., 1991). This is mainly an indirect effect, via alterations in T cell production of IL-2, an important growth and differentiation factor for B cells, IFN-γ, which primes B cells for mitogenesis, and IL-6 (Punnonen and Eskola, 1987; Boyd et al., 1987; Thomson, 1992). However, a direct effect on B cells also occurs (Mongini et al., 1992). Our data showed a decrease in B cell numbers, but not in plasma cell numbers, and we also found no decrease in IL-4, which determines IgE production (DelPrete et al., 1988). This contrasts with the findings reported in VKC (El-Asrar et al., 1996), although this may partly relate to the differences in the duration of CsA treatment (3 weeks versus 3 months). Although AKC is a T cell-mediated disease, it occurs in the context of atopy and there is strong evidence for involvement of type I hypersensitivity inflammation (Tuft et al., 1991; Bonini, 1993). There are conflicting reports regarding the effect of CsA on IgE production, with *in vitro* evidence for inhibition of IgE production (indirectly, via T cell effects) (Okudaira et al., 1986) but failure to reduce serum IgE and clinical type I hypersensitivity responses in patients with AD (Munro et al., 1991). It may be that the precise effect of CsA on IgE production and type I hypersensitivity is influenced by the cytokine context of the disease and it appears that VKC involves Th-2-type T cells whereas in AKC the T cell profile is skewed towards a more Th-1-type (Chapter 4). However, the lack of reduction of IgE-specific plasma cells in VKC (El-Asrar et al., 1996) and in total plasma cell numbers shown here suggests that topical CsA is probably not exerting its role via effects on IgE production.

8.4.4 Effects on granulocytes

Neutrophils were reduced in number but there were no changes in mast cell and eosinophil numbers. CsA is known to reduce the ability of neutrophils to respond to a chemoattractant indirectly through effects on cytokines (Mozzanica et al., 1993). In asthma models, pulmonary eosinophilia was reduced by CsA (Norris et al., 1992; Elwood et al., 1992). It has been suggested that CsA may exert effects on eosinophil numbers via a reduction in IL-5 release from T cells (Borel et al., 1996). There was only a small decrease in IL-5, and the degree of eosinophilia in this group of AKC patients was too low to allow any detectable reduction in eosinophil numbers. However, there is recent evidence that CsA has direct (as well as indirect) effects on

both mast cells and eosinophils, inhibiting their activation, degranulation and inflammatory mediator release (Wershil et al., 1995; Hultsch et al., 1990; Triggiani et al., 1989; Kita et al., 1991; Cirilo et al., 1990), which may be very important in any effects on chronic allergic eye disease. Whether these *in vitro* findings translate to *in vivo* effects is controversial (Borel et al., 1996; Munro et al., 1991).

8.4.5 Conclusion

The beneficial clinical effects of topical CsA in AKC appear to be paralleled by immunomodulatory changes in leukocyte numbers and cytokine expression. The decrease in T cell IL-2 and IFN-γ expression in this study correlates well with the known *in vitro* effects of CsA, but as these cytokines are particularly elevated in AKC (Chapter 4), it might be that topical CsA will have an even more beneficial effect in AKC than that seen in VKC.

Chapter 8: Tables and figures

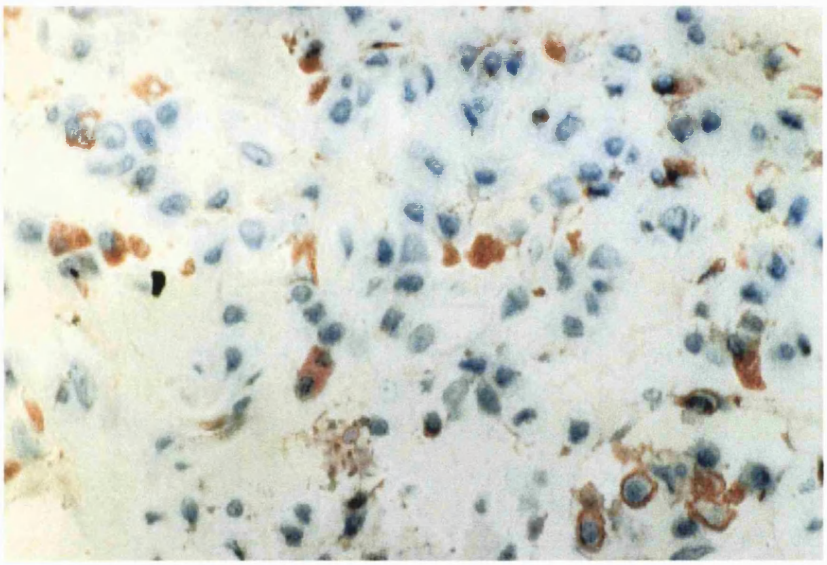


Figure 8.1a. One-colour IHC demonstrating HLA-DR$^+$ cells (x100, AEC).

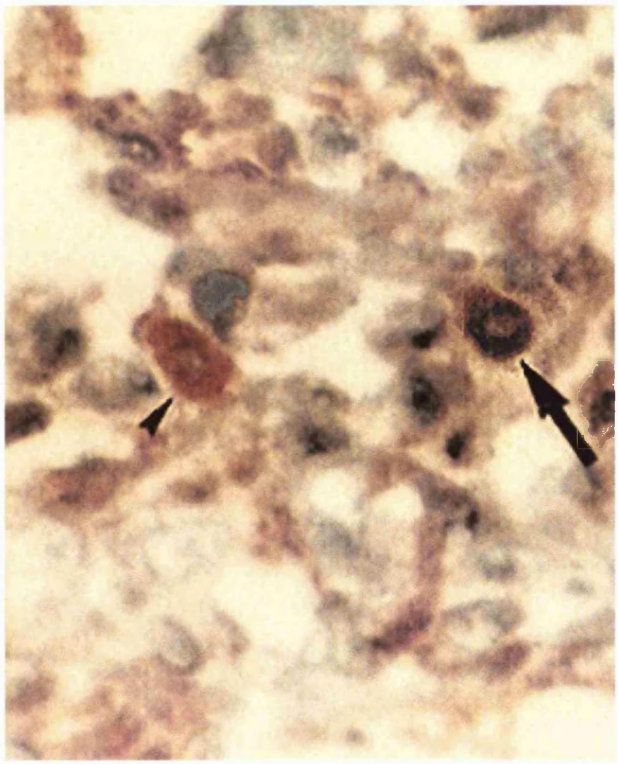


Figure 8.1b. Two-colour IHC demonstrating double-stained CD3$^+$ T cells expressing IL-4 (arrow) and non-CD3 cell expressing IL-4 (arrow head) (x400, AEC, DAB).

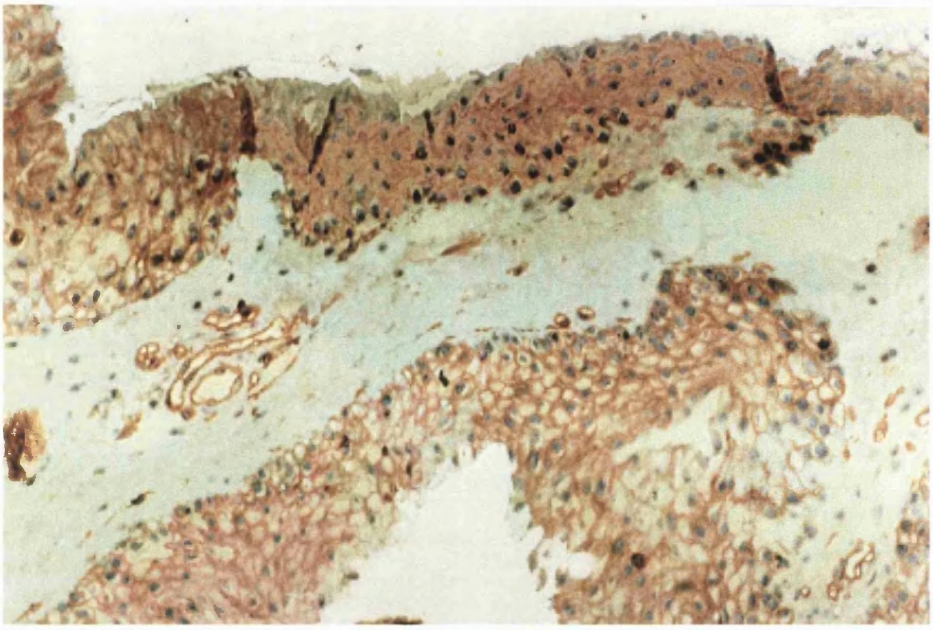


Figure 8.1c. One-colour IHC demonstrating positive staining for ICAM-1 on epithelial cells and vascular endothelium (x100, AEC).

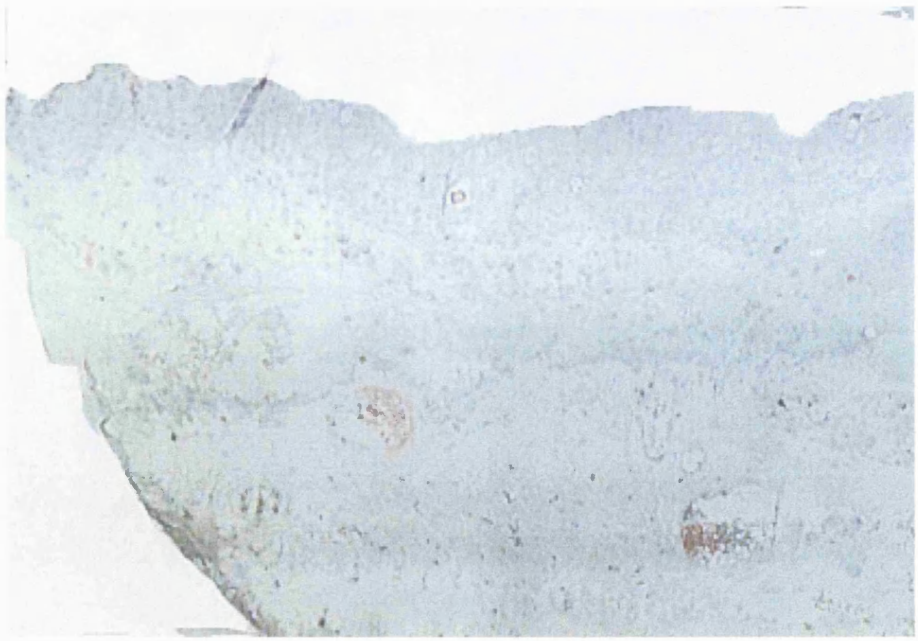


Figure 8.1d. Negative control demonstrating no AEC staining using an irrelevant antibody (x100, AEC).

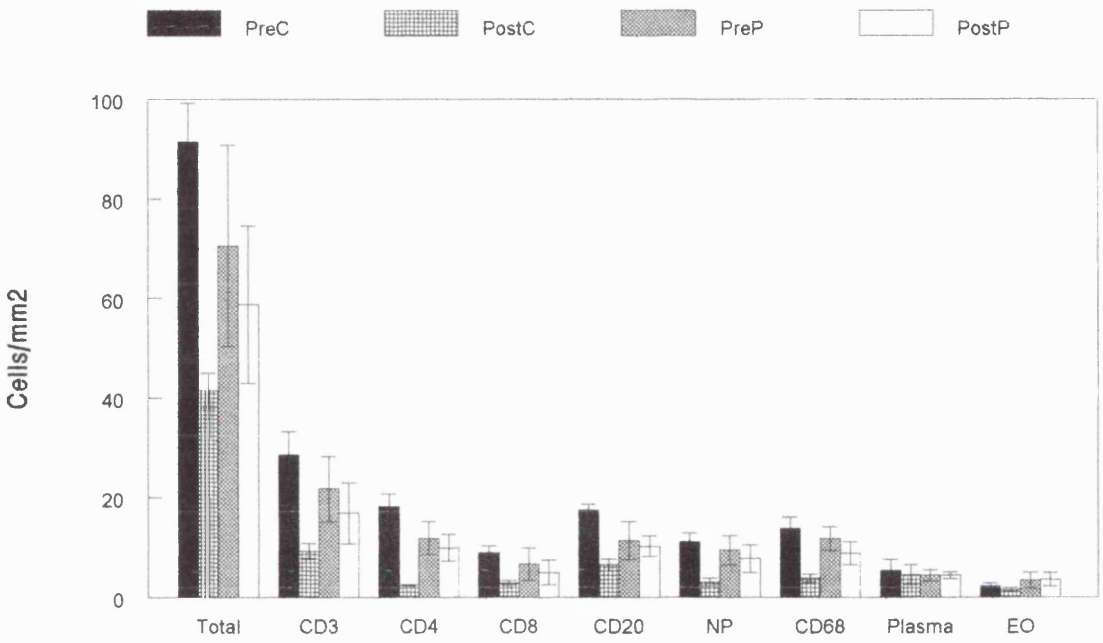


Figure 8.2. Total and individual leukocyte cell counts in the substantia propria before and after CsA and placebo therapy.

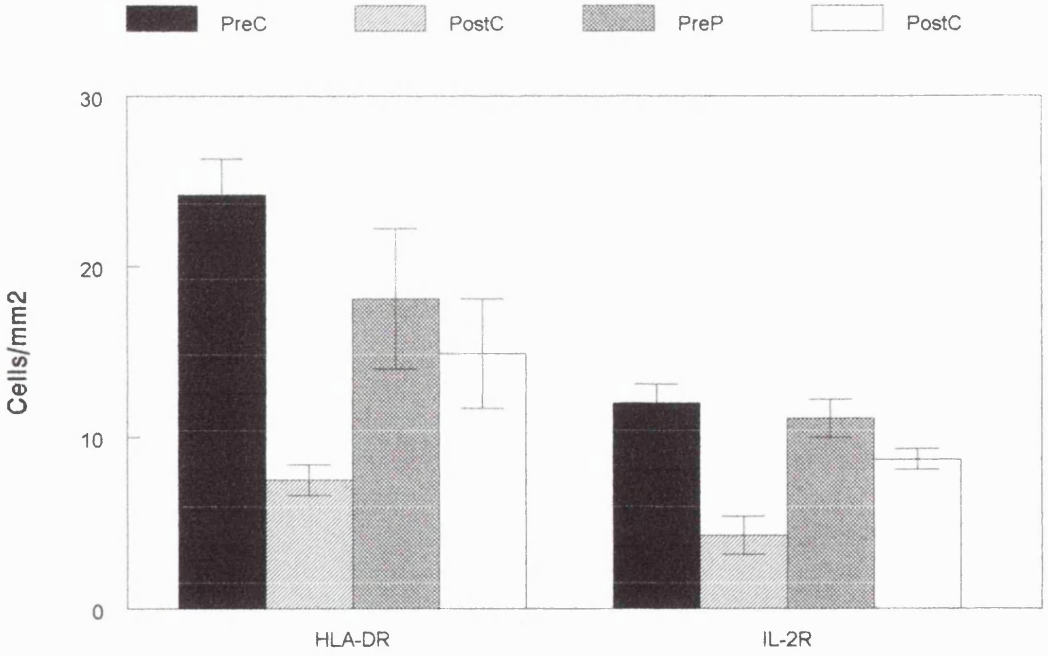


Figure 8.3. HLA-DR$^+$ and IL-2R$^+$ cell counts in the substantia propria before and after CsA and placebo therapy.

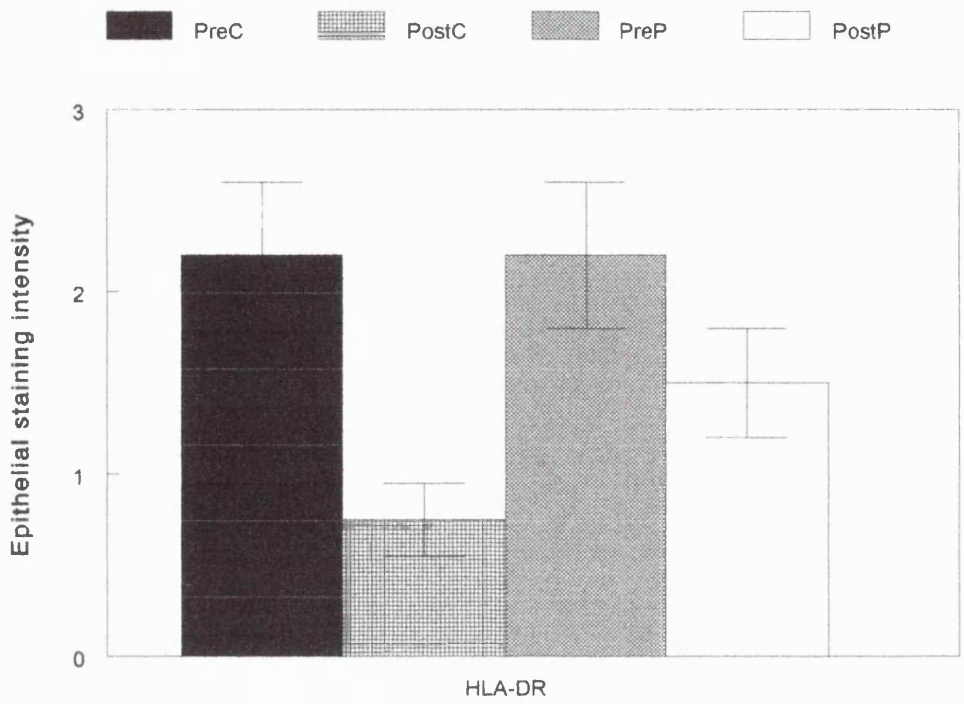


Figure 8.4. Epithelial cell staining grade for HLA-DR before and after CsA and placebo treatment.

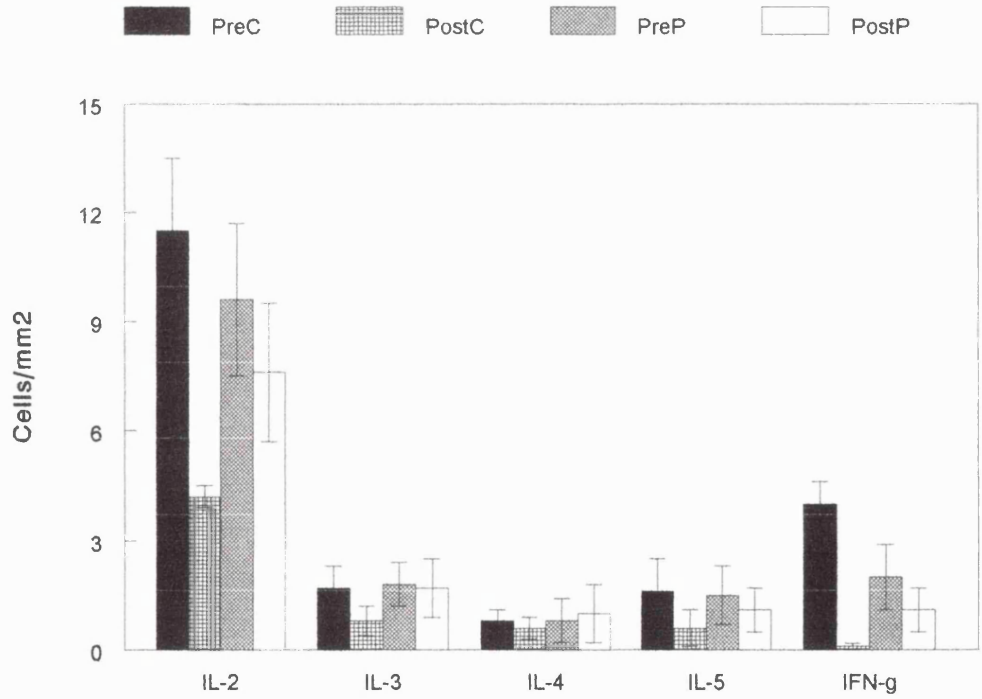


Figure 8.5. Numbers of T cells in the substantia propria staining for cytokine before and after CsA and placebo.

Table 8.1: Primary monoclonal antibodies used in CsA studies

Monoclonal antibody	Specificity	Source
Antibodies to cell surface antigens:		
CD3	T cells	Dako
CD4	T cells subset & some eosinophils	Dako
CD8	T cell subset	Dako
CD20	B cells (not plasma cells)	Dako
NPE	Neutrophils (neutrophil elastase)	Dako
EG-2	Eosinophils (eosinophil cationic protein)	Sera-Lab
AA-1	Mast cells (mast cell tryptase)	Dako
CD68	Macrophages, monocytes	Dako
HLA-DR	MHC class II molecules	Dako
IL-2R (CD25)	Interleukin-2 receptor	Dako
Antibodies to cytokines:		
IL-2	Interleukin-3	Genzyme
IL-3	Interleukin-3	Genzyme
IL-4	Interleukin-4	Genzyme
IL-5	Interleukin-5	Genzyme
IFN-γ	Interferon-gamma	Genzyme

Chapter 9

General discussion and conclusions

9.1 Background

9.1.1 The importance of ocular allergy

Ocular allergic disease is very common and causes much symptomatic misery and, like other allergic diseases, its incidence has progressively increased over recent decades. Although the majority of sufferers will not develop long-term visual sequelae, the less common AKC and VKC carry significant ocular morbidity and, in certain geographical areas (e.g. Mediterranean, Middle East, Africa), VKC is a common, blinding condition. The treatment of AKC and VKC remains difficult due to the side-effects of the most efficacious treatments.

9.1.2 Research in the field of ocular allergy

Until quite recently there was little investigation into the pathogenesis of ocular allergy, particularly compared to systemic allergic diseases such as asthma and AD, and serious study of the pathogenesis of VKC and AKC has only developed during the last 10 years. Despite the relative explosion in research interest into ocular allergy in this time, work on the serious ocular allergies is limited to a few institutions where there is specialist clinical expertise managing a significant number of these patients combined with appropriate research facilities. The main investigators deserve particular mention: Trocme and colleagues (Mayo Clinic, USA) have demonstrated the importance of eosinophils and granule proteins in allergic keratopathy; Romagnani and colleagues (University of Florence, Italy) have shown the importance of T (especially Th2-like) cells in the pathogenesis of VKC; Secchi and colleagues (University of Padua, Italy) continue to show the importance of mediators such as histamine, histaminase and the eosinophil granule proteins in VKC and are currently investigating the mechanisms of conjunctival scar tissue formation; Saiga (Nippon Medical School, Japan), Tabbara (King Saud University, Saudi Arabia) and Ciprandi (University of Genoa, Italy) and colleagues have investigated adhesion molecule mechanisms; Bonini and Bonini (University of Tor Vegali, Rome and University of Naples, Italy) publish on many aspects of ocular allergic mechanisms; Foster and colleagues (Massachusetts Eye and Ear Hospital, USA) have performed wide-ranging research into the clinical manifestations, pathogenesis and treatment of AKC.

In the United Kingdom, Moorfields Eye Hospital is uniquely advantaged for the study of VKC and AKC because of its large group of AKC and VKC patients, its ophthalmic allergic specialists and the facilities and expertise of the Institute of Ophthalmology. This has generated an ocular allergy research group which, unusually, studies the pathophysiology of different cells concurrently, and also publishes work on the clinical presentation and management of such patients. The aim of the current research is to

investigate the leukocyte (eosinophil, T cell and [in conjunction with Southampton University] mast cell) mechanisms and their interactions, and the role of epithelial cells, in AKC and VKC and also to investigate the modifying effects of therapeutic interventions. An area of particular interest is the pathogenesis of sight-reducing corneal damage. The investigations are based on harvesting conjunctival tissue from subjects with these disorders, subjects with a similar, non-cornea-damaging disease, GPC and from normal control subjects. The research in this thesis examined conjunctival specimens by tissue staining, IHC and ISH to investigate the role of T cells, eosinophils and conjunctival epithelial cells in AKC and VKC and to study the clinical and tissue effects of a new therapy for AKC, topical CsA.

9.2 Results of the project

9.2.1 The normal conjunctiva

The first study investigated the leukocyte population of the normal conjunctiva to allow comparison with ocular allergy and other disorders. The normal leukocyte population of the substantia propria was shown to consist of T cells, B cells, macrophages, plasma cells, NK cells, mast cells and neutrophils, but only T cells, macrophages and infrequent B cells and neutrophils in the epithelium. T cells were most frequent and were mostly antigen experienced ($CD45Ro^+$), probably reflecting the heavy antigen exposure of the conjunctiva. Bulbar conjunctiva contained higher numbers of cells and increased T cell activation which may relate to greater antigen exposure. CALT components such as conjunctival IELs and scattered LPLs were shown to have properties similar to equivalent MALT cells in other mucosae, but the presence of organised CALT aggregates appeared to be less common than sometimes stated.

9.2.2 T cells and eosinophils in chronic allergic eye disease

The second part of the thesis examined the role of leukocytes in chronic allergic eye disease.

9.2.2.1 T cells

The functional conjunctival T cell subsets involved in VKC, AKC and GPC were investigated, by determining their cytokine profiles, and compared to normal subjects. Previous *in vitro* work had suggested that Th2-like cells were important in VKC, as they appear to be in other atopic diseases. The results supported the role of Th2-like cells in VKC and GPC. However, AKC showed a different T cell profile suggesting either a mixed population of cells with a shift towards a Th1-like profile, or cells with a wider individual range of cytokine production resembling Th0 cells. These differences

may relate to clinical characteristics and may be exploitable for therapeutic or preventative intervention.

9.2.2.2 Eosinophils

The role of eosinophils in chronic allergic eye disease, particularly with respect to corneal damage, was investigated. No correlation was found between eosinophil numbers and keratopathy, but differences in cell surface antigens suggested that there is greater eosinophil activation in disorders affecting the cornea. Also, the cytokine profile of eosinophils differed in the different disorders, with IL-5 prominent in GPC, IL-3, IL-5, IL-6 and GM-CSF in VKC, and IL-4, IL-8 and GM-CSF in AKC. The activated eosinophils in the disorders with corneal involvement are likely to be releasing their cytokine contents in significant quantities compared with GPC. It appears that eosinophils, like T cells, show different functional subsets in different ocular allergic disorders which are likely to relate to clinical differences including corneal involvement.

9.2.3 Conjunctival epithelial cells

The role of conjunctival epithelial cells in ocular allergy was investigated by examining expression of cell surface antigens and cytokines. Conjunctival epithelial cells express greater ICAM-1 and HLA-DR and this is most marked in disorders involving the cornea. Certain epithelial cell cytokines are upregulated in ocular allergy (RANTES, and IL-8 only in GPC) and there is induction of GM-CSF (particularly in AKC) and of IL-3 in VKC and AKC. Thus, as for T cells and eosinophils, there are variations in epithelial cell pro-allergic and inflammatory functions between the clinical disorders.

9.2.4 The effect of topical CsA in AKC

9.2.4.1 Clinical trial of CsA in steroid-dependent AKC

This trial showed that topical CsA produces clinical benefit in steroid-dependent patients with AKC and allows steroid use to be weaned and often ceased altogether, without serious adverse drug or disease effects. However, CsA caused adverse symptoms (visual blurring and ocular stinging) which make it difficult to tolerate and possibly unacceptable to patients unless they have severe, steroid-dependent disease or are very well-motivated, This, together with difficulties in the manufacture of the drug in eyedrop form, means that its use will remain confined to large, specialist centres, unless a new formulation of the topical drug can be developed.

9.2.4.2 Immunomodulatory effects of topical CsA in AKC

This study showed that CsA produces immunomodulatory conjunctival changes which occur in association with the clinical improvement in AKC, particularly reductions in the number of leukocytes (T cells, B cells, neutrophils and macrophages), a normalisation of CD4/CD8 ratios, a reduction in T cell activation and T cell expression of cytokines (particularly IFN-γ and IL-2). There appeared to be little effect on type I hypersensitivity-related responses. In combination with the results on T cell subsets in AKC and VKC, these results suggest that AKC patients may be more sensitive to CsA than VKC patients.

9.3 Summary of studies and their potential relevance to future clinical practice

The pathogenesis of chronic allergic eye disease is complex, with several cell types generating relevant cytokines, giving them the capability to exert control over each other and over other inflammatory and tissue cells. In addition, each cell type shows functional variations between the disorders which produce corneal damage and those that do not, between each disorder (e.g. AKC vs. VKC) and even between individual patients. The pertinence of such variations in cellular function to clinical characteristics (inter-disease and inter-patient) is unknown.

Exactly how the separate cellular variations interact remains a mystery; whether variations in eosinophil or mast cell cytokines precede and determine those of T cells or vice versa, or whether there is a continuous, complex interaction between all cytokine-secreting cells is not yet known. There are many possible influences on the development of these cellular differences including patient age, genetic background, the nature, amount and timing of antigenic stimulation, the general hormonal and cytokine environment and therapeutic interventions. The presence of cellular variations provide a potential basis for future developments such as the prediction of corneal involvement and enhanced prognostics, improved prevention, better prediction of which therapeutic agents will be most useful in which patients, and hopefully the production of highly-specific but potent therapies (e.g. anti-cytokine agents, anti-eosinophil agents) without the adverse effects of less specific drugs (e.g. steroids). It is even possible to envisage in the future the construction of therapeutic interventions specifically tailored to the disease subtype, the disease complication and even the individual patient, based on cellular variations (for example a cocktail of specific anti-cytokine agents).

9.4 Relevance to systemic allergic disease

9.4.1 Relevance to asthma

Asthma is considered to be the most important and serious allergic disease because of its high (and rapidly increasing) prevalence and because it is associated with significant morbidity and mortality (Peat et al., 1994; Aberg et al., 1996). However, research into the pathogenesis, clinical subtypes and treatment of asthma is inhibited by the inaccessibility of the lower respiratory tract to visualisation, tissue specimen retrieval and drug delivery. Other sites of allergic disease (skin, nose) have been suggested as more accessible areas which can act as models of allergic bronchial inflammation (Vignola et al, 1998; Leung 1995) but the skin is not a mucosal site and more attention has focused on allergic rhinitis.

There are a number of factors which support the use of rhinitis as a model for asthma (Vignola et al., 1998; Durham 1998; Howarth, 1998). The nose constitutes the upper, and the lung the lower, airway and their mucosal linings are continuous and almost identical. The epidemiology of rhinitis and asthma are similar, as are many of the triggering stimuli, the pathological changes, inflammatory mediators and treatments. However, allergic conjunctivitis is another common site of mucosal allergic inflammation which could be used as a model of asthma: allergic conjunctivitis and allergic rhinitis coexist very commonly and there is a strong association between allergic eye disease and asthma (Frankland and Easty, 1971; Allansmith et al., 1977; Tuft et al., 1991; Buckley, 1988a); the conjunctival mucosa is continuous with the mucosa of the upper airway via the lacrimal drainage apparatus although it is stratified (not pseudostratified) epithelium and not ciliated; the conjunctiva shows a two-phase clinical and inflammatory response to allergen challenge like that seen in the lung and the nose (Abelson et al, 1990a; Merayo-Lloves et al., 1996); the immunopathological changes in allergic conjunctivitis parallel those in rhinitis and asthma, with similar overall patterns of leukocyte recruitment, adhesion molecule upregulation and cytokine production (reviewed in Chapter 1); and treatment of rhinitis, conjunctivitis and asthma all involve local and sometimes systemic delivery of similar agents (mast cell inhibitors, steroids) (Vignola et al., 1998; Hingorani and Lightman, 1995). Neither the nose nor the conjunctiva have any equivalent to the bronchial smooth muscle and as models of asthma cannot address questions about bronchoconstriction.

The conjunctiva provides a particularly useful location for the study of mucosal allergic inflammation and the effects of anti-allergic drugs. Mucosal tissue from the eye is more accessible than from the nose for direct observation and sampling and far more accessible than in the lung, where direct mucosal assessment is often impractical or unsafe. In addition, unlike rhinitis, epithelial damage (in the cornea) is an important feature of ocular allergy, as in asthma. In past years, findings from non-ocular allergic

diseases have been extrapolated to the conjunctiva to explain the pathophysiology and to suggest new therapeutic possibilities (Sanderson, 1995). It may be more expedient to extrapolate in the opposite direction.

The findings in our study confirm that there are many similarities between the mucosal inflammation in ocular allergy and that in asthma: the tissue is infiltrated by a mixed cell population, with a high frequency of T cells and also eosinophils and mast cells; eosinophils are activated in association with epithelial cell damage and are producing pro-allergic cytokines; epithelial cells express inflammatory cell surface markers and also produce inflammatory cytokines; Th2-like cells are producing typical pro-allergic cytokines, especially in VKC. The most notable result is that there are variations in the patterns of cell activation, surface marker and cytokine expression between the different ocular allergic syndromes but we do not know the relevance of this to non-ocular atopic disease. Direct visualisation of, and ease of tissue sampling from, the ocular mucosal surface has allowed a more accurate subclassification of allergic disease than in any other tissue. Potentially the classification of ocular allergic disease has parallels with that of systemic atopic disease. There are a number of clinical varieties or subtypes of asthma (atopic versus non-atopic or intrinsic asthma; aspirin-sensitive versus non-aspirin-sensitive asthma; nocturnal versus non-nocturnal asthma; childhood versus adult asthma; exercise-induced versus non-exercise-induced asthma) although these are distinguished from each other in a more indirect manner than in allergic conjunctivitis and strict classification of individual patients can be difficult (Samter and Biers, 1968; Krug et al., 1998; Williams and McNicol 1969; Fabbri et al., 1997). However, there are indications that, as in allergic ocular disease, differences in cells and cytokines can be linked to different clinical subtypes in asthma. Kotsimbos et al. showed that the numbers of GM-CSF receptor mRNA- and protein-expressing macrophages and GM-CSF protein in the bronchial submucosa were significantly higher in intrinsic asthma compared to atopic asthma (Kotsimbos et al., 1997). Kraft et al. showed that bronchial epithelial cell expression of CD51 (α_V integrin, vitreonectin and fibronectin receptor) is significantly increased at night in patients with nocturnal asthma compared with patients with non-nocturnal asthma (Kraft et al., 1998). Sousa et al. demonstrated that there was greater expression of IL-5 by mast cells and eosinophils and expression of GM-CSF by mast cells in bronchial tissue in aspirin-sensitive asthma compared with non-aspirin-sensitive asthma and the same group has previously been shown that the numbers of mast cells and eosinophils but not other leukocytes in lung tissue are higher in aspirin-sensitive patients (Sousa et al., 1997; Nasser et al., 1996). Krug et al. have shown an increase in IL-5 production by bronchial T cells in childhood asthma compared with adult asthma which correlated

with bronchial hyperresponsiveness and peripheral blood eosinophilia (Krug et al., 1998). However, as the last group state, there is little published information on such differences between asthmatic subgroups and more data will be required before comparisons can be drawn with the immunopathological differences between ocular allergy subtypes shown in this thesis.

9.4.2 Relevance to atopic dermatitis

There is an extremely strong association of AKC with AD with most cases of AKC occurring in patients who also have AD. Interestingly, the immunopathogenesis of AD resembles that of AKC and there is evidence for both Th1-and Th2-mediated inflammation in AD. Initially, the evidence seemed overwhelmingly in favour of AD as a Th2-mediated disease similar to asthma. Serum total IgE levels are elevated in more than 80% of AD patients and circulating allergen-specific IgE is present in 85% (Leung, 1995). There is often a peripheral blood eosinophilia, chronic skin lesions contain increased numbers of eosinophils, and eosinophil granule protein levels are elevated in the serum and the skin of AD patients (Bos et al., 1992; Leung, 1995; Leiferman et al, 1985; Kagi et al, 1992). Skin from active AD and from allergen-challenge was reported to show Th2 cytokine predominance with a significant increase in cells expressing IL-4 and IL-5, but not IFN-γ, mRNA (Hamid et al, 1994; Kay et al., 1991; Yamada et al., 1995) and overexpression of GM-CSF and IL-10 has also been demonstrated (Ohman et al, 1995; Bratton et al, 1995). Th2-like T cells predominate in the blood (Nakazawa et al., 1997; Yamamoto et al., 1997). Allergen-specific Th2-like cells were demonstrable in the skin in active AD (Van der Heijden et al., 1991; Van Reijsen et al., 1992). In contrast, recent evidence suggests that Th1-like rather than Th2-like cells are important in the pathogenesis of AD. Clinically and histologically AD is not characteristic of a type I hypersensitivity reaction but is almost indistinguishable from contact dermatitis, a prototypical Th1-cytokine-mediated type IV hypersensitivity reaction (Hauser, 1990). Also, it has proved impossible to correlate the observed allergen-specific type I hypersensitivity phenomena with clinical disease activity (Leung, 1995; Herz et al., 1998). Studies examining the overall cytokine expression in AD skin samples showed that IFN-γ mRNA and protein were expressed at high levels in 80% of patients but only 25% expressed increased IL-4 (Grewe et al., 1994). Moreover, it was IFN-γ, not IL-4, expression which correlated with clinical severity and which decreased after successful therapy (Grewe et al., 1994). It appears that both T cell subsets are important in AD and several authors have proposed that the findings are best explained by differences in chronicity of lesions; that is, in acute AD lesions (characterised by spongiosis, and perivascular infiltration of lymphocytes and macrophages, with few eosinophils) Th2 cells are

involved in the initiation of inflammation and that in chronic AD lesions (characterised by hyperkeratosis, little spongiosis, fibrosis and a macrophage-rich infiltrate) Th1 cells are involved in the persistence of inflammation (Grewe et al., 1998; Herz et al., 1998). Studies of the development of cytokine expression over time using sequential biopsies after atopy patch tests and allergen-challenge support such a hypothesis and have shown that at early time points IL-4 mRNA and protein predominate whereas at later time points expression of IFN-γ mRNA and protein predominate (Grewe et al, 1995; Thepen et al., 1996; Tsicopoulos et al. 1994). In AD skin, acute lesions are characterised by enhanced levels of IL-4 and IL-5 whereas chronic lesions were characterised by increased levels of IL-5 and IFN-γ and less (but still significant) levels of IL-4 (Hamid et al., 1994; Werfel et al, 1996). Also, the majority of house dust mite-specific T cell clones from chronic AD lesions have been shown to produce IFN-γ (Werfel et al., 1996). Thus it appears that there is a biphasic response in AD where early lesions are characterised by Th2 cytokines but established, chronic lesions by a shift towards Th1 profile but with maintenance of some Th2 activity (Herz et al., 1998). This has obvious parallels with the findings in this thesis where VKC is characterised by Th2 T cell cytokines but established AKC by a shift towards Th1 T cell cytokines. It is as yet unknown whether recent-onset AKC resembles acute AD with a more Th2-like profile. Perhaps parallels may be better drawn between VKC (which can progress into AKC) and AKC and early and late AD; in both the skin and the conjunctiva, disease chronicity seems to be associated with a shift towards a Th1-like T cell profile. These conjunctival diseases may provide a useful model for the different stages of AD or alternatively for the differences between asthma, a mainly Th2 disease and AD, a mixed Th1 and Th2 disease.

What may be responsible for the alteration in cytokine profile with time in AD and do equivalent mechanisms underlie the differences between VKC and AKC? IL-12 induces differentiation into Th1-like cells (Wu et al, 1993c) and recent evidence suggests that IL-12 may be important in promoting a more Th1-type profile in established AD. IL-12 mRNA expression has been shown to be increased in chronic AD skin lesions and, in atopy patch test skin lesions, increased expression of IFN-γ was immediately preceded by upregulation of IL-12 (Hamid et al, 1996). In addition, conversion of Th2 to a Th1-like profile by Varicella-Zoster infection of AD skin seems to be associated with IL-12 expression (Fujimura et al., 1997). It has been shown that Th2 cytokines stimulate eosinophils to produce IL-12, and it has been suggested that in early AD, secretion of Th2 cytokines attract eosinophils and stimulate them to secrete IL-12 which causes activation of Th1 cells (Grewe et al., 1998). Macrophages have also been suggested as a possible important source of IL-12 in this situation (Thepen et

al., 1996). However, it is not yet known whether IL-12 is present, associated with eosinophils and macrophages or otherwise, in AKC or VKC conjunctiva.

With regard to differences in the ,properties of other cells such as eosinophils, between asthma and AD or between different subtypes of AD or of asthma, there is almost no information to allow comparison with our findings, although eosinophils are known to be synthesising cytokines in these conditions (Kay et al., 1997). Early results show that chronic AD skin shows greater eosinophil activation, eosinophil numbers and eosinophil IL-5 expression compared with acute AD lesions (Hamid et al., 1994) which suggests further investigation will show more differences similar to those shown in different ocular allergic disorders.

9.4.3 Relevance to the use of CsA in non-ocular allergic disease

Oral CsA is used in both asthma and AD. It has been shown to be beneficial in both disorders, leading to clinical improvement and acting as a steroid-sparing agent (Taylor et al., 1989; Sowden et al., 1991; van Joost et al, 1994; Alexander et al., 1992; Lock et al., 1996; Nizankowska et al., 1995) although relapse usually occurs after discontinuation and it has serious systemic side-effects. Topical application of CsA in asthma and AD has started to be explored. In AD, the topical application of 5 to 10% ointment or gel produced clinical benefit without significant blood levels (Mizoguchi et al., 1992; de Prost et al., 1989) but there are no studies of inhaled CsA in human asthma, although a formulation exists for animal and possible human use (Kon and Barnes 1997).

Sparse information exists regarding the histo- and immunopathological effects of CsA in asthma and AD, except that it seems that in systemic allergic disease as in ocular allergy, type I hypersensitivity mechanisms are little altered by CsA therapy (discussed in Chapter 8). A study of one child with AD who was treated with oral CsA showed a reduction in peripheral blood T cell activation and IL-5 production (Ishii et al., 1996) and another study of two children with AD showed that CsA therapy produced a reduction in IL-4 and increase in IFN-γ in peripheral blood T cells (Campbell and Kemp, 1997) but neither study investigated lesional tissue changes. In an animal model of allergic cutaneous inflammation, systemic CsA, unlike steroid, pretreatment did not have any effect on eosinophil accumulation in the skin (Teixeira et al., 1996). In asthma and the lung, there is slightly more information available on the effects of CsA. In rodent allergen challenge, CsA inhibits bronchial infiltration by eosinophils and lymphocytes (Norris et al., 1992; Chapman et al., 1993; Ceyhan et al., 1998). In human asthmatics, CsA has been shown to inhibit IL-5 mRNA expression in peripheral

blood $CD4^+$ T cells (Mori et al., 1995) and to reduce the late increase in blood eosinophils after allergen challenge (Sihra et al., 1997). In the only study to date of effects in the human lung, 12 months of CsA therapy was shown to reduce T lymphocyte (especially $CD4^+$) infiltration and decrease T cell activation and HLA-DR expression in one asthmatic patient (Redinton et al., 1998). Thus it seems that CsA treatment in asthma and AD produces similar reductions of cell numbers to those occurring in AKC but more detailed results are required to make a comparison with the effects in ocular allergy.

9.5 Future directions

The initial direction of research into the pathogenesis of chronic allergic eye disease in our laboratory will take two directions. The first will be to perform ISH on conjunctival tissue samples to allow confirmation of the *production* of cytokines by eosinophils and epithelial cells demonstrated on IHC. The second line of research is to isolate, purify and culture individual cell types (T cells [recently performed, Calder et al., in press], epithelial cells, eosinophils) to allow direct examination of both phenotype and cytokine production (using a combination of FACS analysis, ELISA of culture supernatants and PCR of mRNA). This will be performed on cells from conjunctival tissue (normal and ocular allergic disease subjects) and compared with peripheral blood leukocytes from the same patients and with each other. Once this is done, we hope to be able to elucidate some of the interactions between individual cell types. We will investigate these interactions in a number of ways; the effect of cell products (from culture supernatants) on other cell's phenotype and function will be examined, and direct cell-cell interactions, such as eosinophil and T cell adhesion to, and migration through, conjunctival epithelial cell layers, will be investigated together with an examination of what factors influence such interactions. In this way, the web of cellular inter-relationships can start to be unravelled.

Systemic allergic and atopic disorders are a major cause of morbidity and even mortality in the developed world. There is an immense amount of ongoing work into the pathogenesis and treatment of these conditions. The work presented here (and the work we intend to perform in the near future) can be repeated in diseases such as asthma, AD and allergic rhinitis to determine whether (as is likely) there are also functional variations in leukocytes and epithelial cells and whether these disorders present different cell profiles from those seen in ocular allergic disease. If extrapolations from the pathogenesis of ocular allergy to systemic allergy are possible, this has important implications for the way in which research into systemic allergic

disease can performed, and may allow at least preliminary investigations into pathogenesis and assessments of new therapies to be performed safely and easily in the conjunctiva.

The final, long-term aim of the research presented in this thesis is to improve patient care by providing a basis for developing more effective and safer treatments, as well as aiding disease and complication prevention in ocular allergic disease. Once determination of cellular variations can be performed on larger groups of patients with chronic ocular allergic diseases, these patients can be followed up long-term and correlation made between these variations and disease outcomes; this will not only provide data to allow better prognostication but may also lead to preventative therapies. The results of our studies suggest certain therapeutic agents (either launched or in development for systemic allergy) could be effective in these diseases, such as anti-cytokines (e.g. dacliximab, an IL-2R monoclonal antibody; suplatast tosilate, an inhibitor of IL-4 and IL-5 release; FK-506, an IL-2 inhibitor; and TRD-96045, an IFN-γ and -α agonist); anti-T cell preparations (e.g. prilixamab, an anti-CD4 monoclonal antibody; peldesine and mycophenolate mofetil, both purine nucleoside phosphorylase inhibitors) and anti-adhesion agents (e.g. anti-ICAM, VCAM-1 and VLA-4 monoclonal antibodies) (Hingorani, 1998). In addition, the results suggest the possibility of developing new agents, such as anti-eosinophil agents (e.g. heparin, anti-granule protein monoclonal antibodies and competitive inhibitors, general inhibitors of eosinophil function) or mixtures of anti-cytokines. All such compounds could be assessed in the *in vitro* models described above (e.g. isolated cell cultures, cell-cell interaction models) before *in vivo* in the eye (animal and then human), with assessment clinically and via tissue examination. However, this will be very much limited by the ability to develop suitable and safe topical preparations for the eye. Of the compounds mentioned here only peldesine has so far been developed in an ophthalmic formulation (in preclinical trials for uveitis and corneal transplant rejection).

It is hoped that the results of the studies presented here and those of the future research undertaken in our facility will significantly aid our understanding of the pathogenesis of chronic ocular allergy and promote the development of better care and prevention in the future for such patients, including those involved subjects who have given so generously of their time and help.

Appendices

Appendix 1a. Normal subjects for the investigation of normal conjunctival leukocyte population

Pt No	Age (years)	Sex	Race	Operation	Medical History
DN1	30	m	C	squint	
DN2	66	f	C	cataract	
DN3	65	f	AC	cataract	
DN4	81	m	C	cataract	
DN5	87	f	A	cataract	
DN6	79	f	C	cataract	
DN7	66	m	A	cataract	
DN8	73	f	AC	cataract	Hypothyroidism
DN9	64	f	C	cataract	
DN10	42	m	C	cataract	Diabetes
DN11	38	f	C	squint	
DN12	68	f	C	cataract	
Subtotal	**63.2 ±5.1 [30-87]**				
DN13*	73	f	C	cataract	Hypertension
DN14*	45	f	A	cataract	
DN15*	69	m	A	cataract	
DN16*	46	f	C	cataract	
DN17*	18	m	C	squint	
DN18*	63	m	C	cataract	Hypertension
DN19*	65	m	AC	cataract	IHD
DN20*	47	f	C	cataract	
Subtotal	**53.2 ±6.4 [18-73]**				
Mean / total	59.3 ±4.0 [18-87]	12f, 8m			

* = both tarsal and bulbar biopsies studied

Abbreviations for appendices:
C = Caucasian white; A = Asian; AC = Afro Caribbean; IHD = ischaemic heart disease; Urt = urticaria; AD = atopic dermatitis; HF = hayfever; Asth = asthma; PR = perennial rhinitis; FA = food allergy.
Rx = treatment; SCG = sodium cromoglycate; NS = nedocromil sodium; Dex = dexamethasone; Pred = prednisolone; FML = fluoromethalone; Ac-cyst = acetyl-cysteine.

Appendix 1b. Details of normal control subjects in comparison with ocular allergic disease

Patient Number	Age (years)	Sex	Race	Operation	Medical History
ISHN1	24	m	C	squint	
ISHN2	53	m	A	cataract	
ISHN3	15	m	C	squint	
ISHN4	13	m	C	squint	
ISHN5	11	f	C	squint	
ISHN6	14	f	C	squint	
ISHN7	5	f	C	squint	
ISHN8	14	f	AC	squint	
ISHN9	32	m	C	squint	
ISHN10	60	f	AC	cataract	IHD
Subtotal	**24.1 ±5.9 [5-60]**	**5f, 5m**			
MN1	19	f	C	squint	
MN2	44	m	A	cataract	Diabetes
MN3	32	m	C	squint	
MN4	62	f	A	cataract	
MN5	85	f	C	cataract	Hypertension
MN6	48	f	C	cataract	
MN7	46	f	C	squint	
MN8	78	m	C	cataract	IHD
MN9	80	f	A	cataract	
MN10	28	m	C	squint	
Subtotal	**55.2±7.3 [19-85]**	**6f, 4m**			
Mean / total	**38.1±5.6 [5-85]**	**11f, 9m**			

Appendix 2. Details of subjects with ocular allergic disease: 2a. Subjects with GPC

Patient Number	Age (yrs)	Sex	Race	Age disease onset (yrs)	Duration disease (yrs)	Atopic disease	Rx before washout	Medical History
ISHGPC1	22	f	C	19	2	Urt, AD	SCG	
ISHGPC2	39	f	C	31	8	None	Nil	High myopia
ISHGPC3	32	f	A	18	14	None	Nil	Keratoconus
ISHGPC4	68	m	C	63	5	HF	Nil	
ISHGPC5	26	f	C	20	6	AD, Asth, PR, HF, Urt, food allergy	Nil	
ISHGPC6	24	f	C	19	5	AD, PR, HF, Urt, food allergy	SCG	
ISHGPC7	28	f	AC	16	12	Asth, PR, HF	SCG	Keratoconus
ISHGPC8	44	f	C	44	0.5	Asth, PR, HF, Urt, AD	Nil	
Subtotal	**35.4 ±5.4 [22-68]**			**28.7 ±5.9 [16-63]**	**6.5 ±1.6 [0.5-14]**			
MGPC1	59	f	C	54	5	AD, HF	Nil	
MGPC2	31	m	A	28	3	None	Lubricants	
MGPC3	20	f	C	18	2	None	SCG	
MGPC4	53	m	C	45	8	None	Nil	
MGPC5	40	m	C	35	5	Asth, HF	Nil	
MGPC6	23	m	C	22	1	HF, PR	Nil	
MGPC7	73	m	C	69	4	AD	SCG, lubricants	
MGPC8	22	m	C	2	1	None	SCG	Aphakia
MGPC9	68	f	C	65	3	None	Nil	IHD, hypertension, hypothyroidism
MGPC10	29	m	C	29	0.5	AD	Nil	Keratoconus
Subtotal	**41.8±6.3 [20-73]**	**3f**		**36.7 ±6.8 [2-69]**	**3.2 ±0.7 [0.5-8]**			
Total	**38.9±4.2 [20-73]**	**10f**		**33.2±4.5 [2-69]**	**4.7±0.9 [0.5-14]**			

Appendix 2. Details of subjects with ocular allergic disease. 2b. Subjects with VKC

Patient Number	Age (years)	Sex	Race	Age disease onset (yrs)	Duration disease (yrs)	Atopic disease	Rx before washout	Medical history
ISHVKC1	24	f	AC	22	2	AD, food allergy	SCG	
ISHVKC2	25	m	C	3	22	Asth, PR, hf, URT, AD	SCG, Dex	
ISHVKC3	18	m	AC	13	5	None	SCG	
ISHVKC4	18	f	C	11	7	None	SCG	
ISHVKC5	15	m	C	8	7	None	NS, Luxazone	
ISHVKC6	15	m	C	6	9	Asth, PR	NS, Luxazone, lomudal	
ISHVKC7	15	f	C	5	10	Asth	SCG, Pred	
ISHVKC8	19	m	A	3	16	None	SCG, Pred	
Subtotal	**18.6 ±1.4 [15-25]**			**8.9 ±2.2 [3-22]**	**9.7 ±2.3 [2-22]**			
MVKC1	25	m	C	10	15	HF, PR	SCG	
MVKC2	18	f	A	12	6	Asth, AD	SCG, Ac-cyst	
MVKC3	18	m	AC	11	7	HF	Lubricants	
MVKC4	23	m	C	9	14	Asth	SCG, Dex, Ac-cyst	
MVKC5	19	m	C	13	6	HF	SCG	
MVKC6	23	f	C	11	12	None	SCG	
MVKC7	15	m	C	8	7	Asth, AD	SCG, Dex	
MVKC8	29	m	AC	27	2	PR, Urt, AD	SCG	
MVKC9	19	m	C	15	4	Asth, HF, AD	SCG, Pred	
MVKC10	12	m	C	7	5	Asth, AD, HF	SCG, Pred	
Subtotal	**20.0 ±1.6 [12-29]**	**2f**		**12.3 ±1.8 [7-27]**	**7.8 ±1.4 [2-15]**			
Total	**19.4±1.0 [12-29]**	**5f**		**10.8±1.4 [3-27]**	**8.6±1.2 [2-22]**			

Appendix 2. Details of subjects with ocular allergic disease. 2c. Subjects with AKC

Patient Number	Age (yrs)	Sex	Race	Age disease onset (yrs)	Duration disease (yrs)	Atopic disease	Rx before washout	Medical History
ISHAKC1	29	f	C	1	28	Asth, AD, PR, HF, Urt	None	
ISHAKC2	25	m	A	5	20	Asth, PR, HF, Urt, AD, FA	SCG, Dex	
ISHAKC3	22	f	AC	5	17	Asth, AD, PR, HF, Urt, FA	SCG, Pred	
ISHAKC4	46	m	C	2	44	Asth, AD	SCG	
ISHAKC5	42	m	C	6	36	Asth, PR, HF, Urt, AD	Dex	
ISHAKC6	39	f	C	19	20	Asth, AD	SCG	
ISHAKC7	29	f	C	13	16	Asth, AD, PR, HF, FA	SCG, Pred	
ISHAKC8	21	f	AC	10	11	Asth, AD, HF	SCG	
Subtotal	**31.6 ±3.3 [21-46]**			**7.6 ±2.1 [1-19]**	**24 ±3.9 [11-44]**			
MAKC1	29	f	C	21	8	AD, HF	SCG, Pred	
MAKC2	28	m	C	19	9	AD	NS, Pred	
MAKC3	25	m	C	20	5	Asth, AD, HF, PR	Pred	
MAKC4	21	f	A	12	9	Asth, AD	SCG, FML	
MAKC5	21	m	C	19	2	Asth, AD, HF	SCG	
MAKC6	29	m	C	18	9	AD	SCG, Dex	
MAKC7	26	m	AC	14	12	Asth, AD, HF, Urt	NS, Dex	
MAKC8	22	m	C	9	13	Asth, AD, PR	SCG, Pred	
MAKC9	46	m	C	24	22	Asth, AD	NS, Dex	
MAKC10	32	m	C	18	14	Asth, AD, PR, HF	SCG, FML	
Subtotal	**27.9±2.3 [21-46]**	**3f**		**17.4 ±1.4 [9-24]**	**10.3 ±1.7 [2-22]**			
Total	**29.5±1.9 [21-46]**	**7f**		**13.0±1.7 [1-24]**	**16.4±2.5 [2-44]**			

Appendix 3. Subjects in trial of CsA in AKC

3a. Subjects with steroid-dependent AKC in clinical trial of CsA

Patient No. (trial number)	Age (yrs)	Sex	Race	Atopic disease	Rx	Medical History
P1 (2A)	61	f	A	Asth, AD, PR	FML, SCG	Diabetes insipidus
P2 (3A)	29	m	C	AD	Pred 0.1%	None
P3 (6A)	29	m	C	AD, Asth, HF, Urt	Dex, SCG	None
P4 (10A)	29	f	C	AD, HF	Pred 0.1%, SCG	None
P5 (15A)	28	m	A	AD, HF	Dex, SCG	None
P6 (16A)	26	f	C	Asthma, AD, HF, FA	Pred 0.3%, NS	Keratoconus
P7 (17A)	31	f	C	Asth, AD, HF	Pred 0.3%, SCG, lubricants	None
P8 (18A)	59	m	C	Asth, AD, HF	Pred 0.3%, SCG	None
P9 (24A)	29	f	C	AD, Urt	FML, Lodoxamide	Keratoconus
Sub	**35.7±4.6 [26-61]**	**5f, 4m**				
C1 (1B)	24	m	A	AD, Asth	Dex, SCG	None
C2 (4B)	36	m	C	AD	Pred 0.3%, SCG, Ac-cyst	Syringomyelia
C3 (5B)	66	f	AC	AD	FML, Ac-cyst, simple ointment	Hypertension
C4 (7B)	25	f	AC	Asth, AD, HF	Pred 0.3%, SCG	Keratoconus
C5 (12B)	48	m	C	Asth	Dex, NS	Hypertension
C6 (13B)	33	m	C	Asth, AD, HF	FML, SCG	None
C7 (14B)	25	m	A	AD, FA	FML, SCG	None
C8 (19B)	18	f	C	AD, HF, PR	Pred 0.3%, SCG	None
C9 (20B)	33	m	C	AD, Asth, Urt	Pred 0.3%, NS, Ac-cyst	None
C10 (21B)	47	f	C	Asth, AD, HF	Dex, SCG, retinoic acid	None
C11 (23B)	24	m	A	Asth, AD	Dex, SCG	Keratoconus
C12 (25B)	28	m	C	AD, HF, PR	Pred 1%, NS	None
Sub	**33.9±3.9 [18-66]**	**4f, 5m**				
Total	**34.7±2.9 [18-66]**	**9f, 12m**				

3b. Subjects with AKC in investigation of immunomodulatory effects of CsA

Patient Number	Age (yrs)	Sex	Race	Atopic disease	Rx (before washout period)	Medical History
MTAKC 11	26	m	A	AD	FML, SCG	None
MTAKC9	22	m	C	AD, Asth, HF	FML, SCG	None
MTAKC8	47	m	C	Asth, AD	Dex, NS	None
MTAKC16	28	m	C	AD	Dex	None
CsA subgroup	**30.7 ±5.5 [22-47]**	**4m**				
MTAKC2	29	m	C	AD	Dex, SCG	None
MTAKC15	22	f	C	Asth, AD, HF	Pred 0.3%, SCG, lubricants	None
MTAKC3	31	m	C	AD	SCG	None
MTAKC4	61	f	AC	AD, Asth	Pred 0.3%, SCG	None
Placebo subgroup	**35.7 ±8.6 [22-61]**	**2m**				
Total	**33.2 ±4.8 [22-61]**	**6m, 2f**				

Appendix 4. Primary mouse anti-human monoclonal antibodies used in IHC.

Monoclonal antibody	Specificity	Source	Dilution
CD3	T cells	Dako Ltd, High Wycombe, UK	1:100
CD4	T cell subset & some eosinophils	Dako	1:10
CD8	T cell subset	Dako	1:20
CD45Ro	Memory T cells	Dako	1:200
TCRγδ	T cell receptor γδ	T Cell Science,	1:20
HML-1 (Ber-ACT8)	Human mucosal lymphocyte antigen-1	Dako	1:100
CD20	B cells (not plasma cells)	Dako	1:50
NPE	Neutrophils (neutrophil elastase)	Dako	1:1000
EG-2	Eosinophils (eosinophil cationic protein)	Sera-Lab, Sussex, UK	1:200
AA-1	Mast cells (mast cell tryptase)	Dako	1:100
CD68	Macrophages, monocytes	Dako	1:100
CD57	Natural killer cells	Becton-Dickinson, Oxford, U.K.	1:20
CD25	IL-2 receptor	Dako	1:50
HLA-DR	MHC class II molecule	Dako	1:5000
CD54	ICAM-1	Dako	1:5000
AE-1	Epithelial cell cytokeratin	ICN Pharmaceuticals, Thame, U.K.	1:200
CMV	Cytomegalovirus	Dako	1:50

IL-2	Interleukin 2	Genzyme Diagnostics, Cambridge, MA, USA	1:1000
IL-3	Interleukin-3	Genzyme Diagnostics	1:1000
IL-4	Interleukin-4	Genzyme Diagnostics	1:1000
IL-5	Interleukin-5	Genzyme Diagnostics	1:1000
IL-6	Interleukin-6	Genzyme Diagnostics	1:1000
GM-CSF	Granulocyte-monocyte colony stimulating factor	Genzyme Diagnostics	1:5000
RANTES	Regulated upon Activation, normal T cells expressed and secreted	R&D systems, Abingdon, UK	1:500
IL-8	Interleukin-8	Genzyme Diagnostics	1:200
TGF-β	Transforming growth factor-β	Genzyme Diagnostics	1:1000
TNF-α	Tumour necrosis factor-α	Genzyme Diagnostics	1:1000
IFN-γ	Interferon-gamma	Genzyme Diagnostics	1:100

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