UNIVERSITY OF SOUTHAMPTON

THE ROLE OF THE MAST CELL IN SEASONAL ALLERGIC CONJUNCTIVITIS

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Doctor of Philosophy

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February 2002

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ABSTRACT

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ABSTRACT

The eye affords a unique opportunity to study the mechanisms of allergic disease and the conjunctiva forms the largest part of the ocular surface. Contact with airborne allergens in susceptible individuals can result in a local conjunctival hypersensitivity response characterized by IgE-mediated activation of mast cells through FcεR1. The subsequent release of both preformed and newly synthesized mediators of inflammation leads to itch, vasodilation, oedema and reflex lacrimation which together, comprise the condition known as seasonal allergic conjunctivitis (SAC) to pollen. The mast cell is well recognized as a central effector cell in allergic reactions, but its cell biology remains poorly understood. The aims of this thesis were, therefore, to investigate the tissue changes associated with SAC, the capacity of mast cells to regulate ocular hypersensitivity in human subjects and to gain new knowledge into the regulation of mast cell function.

Evidence is presented of conjunctival mast cell accumulation in SAC in the absence of increased numbers of eosinophils or neutrophils. By examining thin sections of conjunctiva using immunohistochemistry and *in-situ* hybridization, immunoreactivity for a range of cytokines known to play key roles in the pathophysiology of allergic diseases is demonstrated. Furthermore, these cytokines are well recognized to selectively upregulate cell adhesion molecules critical to the recruitment of leucocytes to areas of allergic inflammation. These findings support the hypothesis that mast cell degranulation can drive the conjunctival late phase response (LPR) in the absence of the activation of other inflammatory cells and thus provide a link between the type I hypersensitivity reaction to pollen and the clinical disease of SAC. This hypothesis was tested using conjunctival allergen challenge to generate a LPR in human subjects followed by the recording of symptom scores and study of the tissue cell and cytokine changes.

These data provide evidence that the conjunctival mast cell is well positioned to orchestrate the early immune response to allergen prior to leukocyte recruitment and activation. Mast cells, however, are heterogeneous and in man, are typically described according to their content of serine proteases. To determine whether this distinction extended to a functional heterogeneity based on cytokine content, the distribution of the T_H2-like cytokines, IL-4, IL-13, IL-5 and IL-6 between mast cell subtypes was investigated.

The tissue regulation of mast cell growth, development, function and survival is poorly understood, but stem cell factor (SCF) is known to play a major role. This thesis reports that human mast cells are themselves a source of this cytokine. This key finding provides novel evidence that a mechanism exists to regulate the biology of this important and ubiquitous cell in an autocrine manner.

TABLE OF CONTENTS

LIST OF FIGURES

APPENDIX 3

LIST OF TABLES

LIST OF ACCOMPANYING MATERIAL

Seasonal allergic conjunctivitis is accompanied by changes in mast cell number in the absence of leukocyte infiltration.
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ACKNOWLEDGEMENTS

I thank William Roche for his supervision in the lab and encouragement. William's academic rigor has provided me with an invaluable training and solid foundation in science. James McGill imparted to me his indomitable enthusiasm and energy and I thank him also for his clinical supervision. I am grateful to Shaoli Zhang for her patient teaching in molecular biology and her collaboration with the work on SCF. Steven Holgate illuminated the research with his clarity of thought and Tom McDonald gave generously of his time and guidance. The occupants of room LE71 opened my eyes to the world of research scientists. The Medical Research Council of Great Britain made this work possible by funding my position and research expenses. I especially thank the participating subjects who showed a true generosity of spirit and support of scientific research.

Without the love and support of Claire there would be no thesis and I am deeply grateful to her. For being my sources of joy and inspiration I thank Guy and Henry.

ABREVIATIONS

AEC	3-Aminoethylcarbazole
CAM	Cell Adhesion Molecule
CAC	Conjunctival Allergen Challenge
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
CD	Cluster of Differentiation
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
FcεR1	High affinity IgE receptor on mast cells and basophils
FITC	Fluorescein Isothiocyanate
GMA	Glycolmethacrylate
IL	Interleukin
ISH	*In-situ* hybridization
IHC	Immunohistochemistry
mRNA	Messenger Ribonucleic Acid
mAb	Monoclonal Antibody
NBT-BCIP	Nitro-Blue Tetrazolamine 3-Bromo-4-Chloro-1-Phosphate
r.t.	Room Temperature

CHAPTER 1

Introduction

Seasonal allergic conjunctivitis (SAC) is a common, unpleasant condition of the eye during the hayfever season. In common with allergic rhinitis, asthma and eczema, SAC is a condition in which IgE is thought to play a central role. In susceptible individuals the deposition of allergen on the mucous membrane of the eye leads to the symptoms and signs of SAC. This abnormal response is mediated by cells of the immune system and their released products.

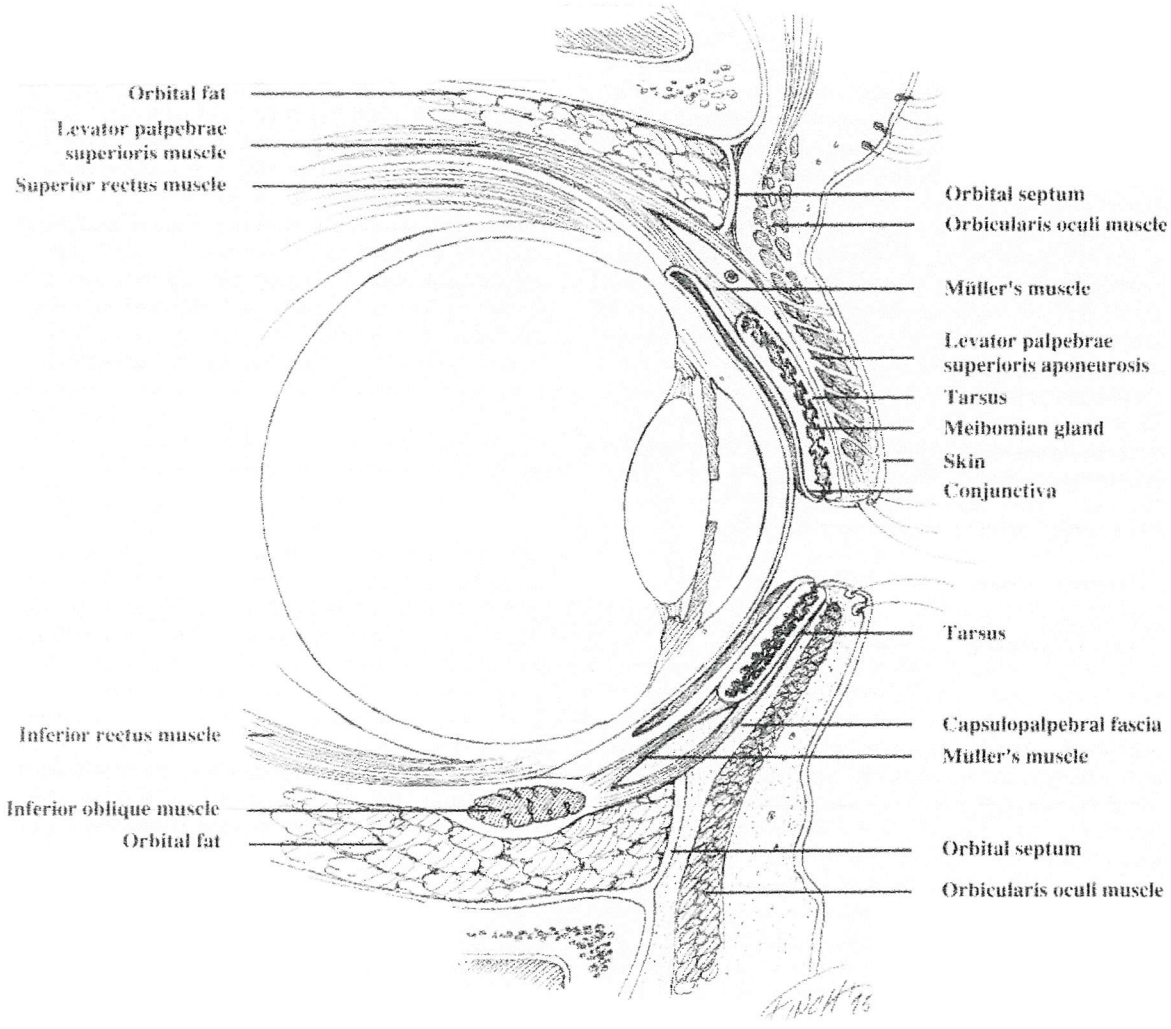
1.1 Ocular anatomy

1.1.1 Overview

The eye is an extension of the central nervous system and functions as the sensory organ of the visual system. It is surrounded by the bony orbit except anteriorly, where it is bound by an extension of periosteum called the orbital septum, and the eyelids. The orbital septum separates the contents of the orbit from the connective tissue of the eyelids. The conjunctiva is a thin, translucent mucous membrane that joins the eyelids to the eyeball. The globe of the eye can be considered as two segments of different radii of curvature superimposed on one another. The smaller anterior compartment has a radius of curvature of approximately 8mm, is filled with liquid, transparent, aqueous humour and is bounded anteriorly by the cornea and posteriorly by the lens (Figure 1.1). The posterior segment has a radius of curvature of about 12mm, is filled with a transparent collagen gel, vitreous humour, and is bounded anteriorly by the crystalline lens and elsewhere by retina (Figure 1.2). The three major coats of the eye from the outside are; the integument of sclera and cornea, the vascular pigmented uveal tract and the neurosensory retina. The sclera which forms the posterior five-sixths becomes continuous with the transparent, colourless cornea anteriorly at the corneo-scleral limbus. The vascular uveal tract forms from back to front, the choroid plexus, the ciliary body and the iris diaphragm. The

FIGURE 1.1

The gross anatomic relations of the conjunctiva

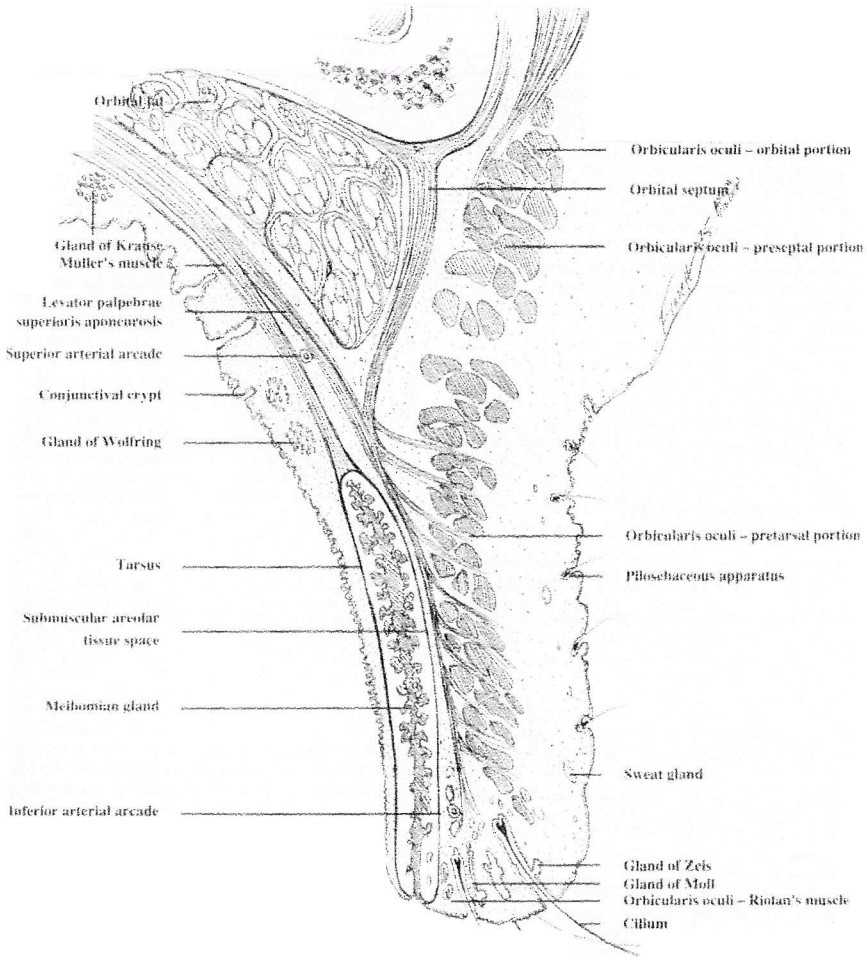


Legend

The conjunctiva forms a sac lining the undersurface of the eyelids and reflected forwards in the fornices to cover the globe of the eye.

FIGURE 1.2

The gross anatomy and relations of the tarsal conjunctiva



Legend

Glands of Krause and Wolfring (see lines) function as accessory lacrimal glands, the tarsal conjunctiva is closely applied to tarsal plate and is limited anteriorly by the mucocutaneous junction

1.1.2 Conjunctival anatomy

Extending from the mucocutaneous junction of the lid margin, the conjunctiva lines the undersurface of the eyelids and is reflected forward in the fornices, a continuous, annular cul-de-sac divided artificially into four regions; marginal, tarsal, bulbar and forniceal. The conjunctiva becomes confluent with the corneal epithelium at the corneoscleral limbus (Figure 1.1). The parts of the conjunctiva are named according to the structures they approximate, so that the marginal conjunctiva extends from the mucocutaneous junction at the lid margin to the subtarsal groove, the tarsal portion overlies the tarsal plate and the bulbar conjunctiva covers the globe of the eye. Fibrous septae adhere the vascular tarsal conjunctiva to the tarsal plate restricting the conjunctival mobility at this site and limiting the spread of oedema during inflammation. The remaining bulbar conjunctiva however, is less firmly attached to the underlying tissue which allows for movement with the eyelids and eyeball. Conjunctiva shares features in common with skin and respiratory mucosa. It is continuous with the respiratory mucosa of the upper airway through the nasolacrimal canal and is also exposed to allergens and irritants. Although sharing exposure to ultra-violet B radiation from sunlight and histological features with skin, conjunctiva is non-keratinised and therefore hydrophilic, and is non-pigmented in Caucasians.

Tears are produced by the lacrimal gland situated in the superolateral part of the orbit. This gland opens directly into the superior conjunctival fornix. Secretion of tears is supplemented by the accessory lacrimal glands of Krause in the fornices and Wolfring in the tarsal and palpebral conjunctiva (Figure 1.1). The tear film, conjunctiva, cornea and lids forms the ocular surface and may be considered to function as a unit [Tseng et al. 1997]. Tears are comprised of a mucous layer continuous with the epithelial glycocalyx, an aqueous layer forming the bulk of the tear film produced by lacrimal secretion, and a lipid layer secreted by tarsal meibomium glands. Tears bathe the conjunctival and corneal surface maintaining a stable refractive surface for the cornea, wetting the ocular surface and removing potential pathogens during blinking. Important tear proteins include: lysozyme, β-lysin and lactoferrin which are bacteriocidal and bacteriostatic; all classes of immunoglobulins; the entire complement cascade and the cysteine proteinase inhibitors, cystatin S and tear lipocalin [Gachon et al. 1998]. In addition to immunological

protection, the tear fluid also contains important growth and nutritional factors vital for the normal proliferation and differentiation of the ocular surface epithelium. These factors include epidermal growth factor (EGF) [Ohashi et al. 1989], transforming growth factor-β (TGF β) [Gupta et al. 1996] , hepatocyte growth factor (HGF) [Li et al. 1996a] and vitamin A [Ubels et al. 1986]. Loss of these factors in various disease states may lead to abnormal epithelial differentiation which may amplify the pathological effects of surface desiccation. Lacrimal gland tears also expose this large surface area to solublized allergen and cytotoxic mediators released by the inflamed conjunctiva. Prolonged contact of these mediators may result in cell damage or death with resultant fibrosis. If this process affects the cornea, visual loss will result through loss of transparency. Damage to the conjunctiva may result in an unstable ocular surface through secondary dysfunction of the tear film which in turn may lead to corneal pathology.

1.1.2.1 Histology

Epithelium

The non-keratinized epithelium of the tarsal conjunctiva begins posterior to the keratinized squamous epithelium of the skin at the mucocutaneous junction. The two to five cell layer thick tarsal conjunctival epithelium changes from squamous to cuboidal towards the fornix. Mucin secreting goblet cells in apocrine or holocrine glands are found in increasing number past the subtarsal groove where they line invaginations of the conjunctiva known as crypts of Henle. Their density varies between 1000 and 56,000 cells mm^{-2} and they are found in maximal numbers in the tarsal and inferonasal conjunctiva and over the plica semilunaris [Ralph 1975]. They are formed basally and migrate to the surface although the source of their precursor stem cell population is not known. Epithelial cells attach to each other and to goblet cells by desmosomes. The basal cells attach to a thin, partly discontinuous and undulating basal lamina by hemidesmosomes. The basement membrane zone does not show immunoreactivity for immunoglobulins or complement in normal subjects. Superficial epithelial cells become flatter from tarsal to forniceal conjunctiva and form the six to nine layer irregular stratification of the bulbar conjunctiva. At the anterior border of the superficial cells, junctional complexes link adjacent cells forming a semipermeable lipophillic membrane. These cells show 0.5-1μm tall microvilli which may fuse to form microplicae. Adherent to these structures is a glycocalyx of long chain glycoproteins secreted by the cellular rough endoplasmic

reticulum and secreted by the Golgi apparatus. This structure allows wetting of the hydrophobic surface and is bound to overlying goblet cell mucin which may form separate layers with the aqueous tear film and overlying meibomium secretion, or may span the tear film. The epithelium at the limbus is between seven and ten layers thick and forms the papillae of the radially arranged palisades of Vogt. There is a gradual transition to the stratified squamous epithelium of the cornea. Melanocytes may be present in the epithelium throughout the conjunctiva. Langerhans cells have been observed in the tarsal conjunctival epithelium [Bron et al. 2000].

Substantia propria

The conjunctival submucosa comprises a superficial lymphoid layer or lamina propria, and a deep fibrous layer by which it is attached to the tarsal plate and fascia covering the globe. The loose connective tissue of the superficial layer contains a 50-70μm thick layer of lymphocytes. Aggregates of T and B lymphocytes are not present in uninflamed normal conjunctiva in infants but may be present in adults in the form of conjunctival associated lymphoid tissue. The deeper fibrous layer contains blood vessels, nerves, lymphatic vessels and glands of Krause.

1.1.2.2 Blood Supply

The tarsal conjunctiva, fornix and posterior bulbar conjunctiva share their arterial supply with the eyelids from the peripheral and marginal tarsal arcades. Ascending branches from these arcades form the posterior conjunctival arteries which anastamose with anterior conjunctival arteries about 4mm from the limbus. The bulbar conjunctiva is supplied by the anterior conjunctival arteries derived from the episcleral arterial circle. This is formed by the deep and superficial saggital system derived from the ophthalmic artery. Fenestrated conjunctival capillaries allow for rapid accumulation of plasma constituents during inflammation. Conjunctival swelling is limited by the nature of the adhesion between the fibrous part of the substantia propria and the underlying tissue. Venous drainage from the palpebral conjunctiva follows the post-tarsal veins of the eyelids. The perilimbal venous circle drains blood from the limbus and anterior conjunctival veins which then drain into the veins draining the rectus muscles.

1.1.2.3 Lymphatic Drainage

An episcleral plexus drains small superficial vessels into larger channels in the deep substantia propria. These join the lymphatic drainage of the eyelids and drain laterally to the superficial parotid nodes and medially to the submandibular lymph nodes.

1.1.2.4 Nerve Supply

Sensory innervation of the conjunctiva is provided mainly by the nasociliary, frontal and lacrimal branches of the ophthalmic division of the trigeminal nerve. The infraorbital branch of the maxillary division innervates a small area of inferior fornix and palpebral conjunctiva. Nerve endings form a plexus in the superficial substantia propria and pass forward unmyelinated to the basal conjunctival epithelial cell layer.

1.2 Hypersensitivity

Hypersensitivity is the term used to describe an adaptive immune response to an antigen not associated with a pathogen [Lichtenstein 1993]. Hypersensitivity reactions also includes those to transplanted tissue and to self-antigens. Immediate or type I hypersensitivity responses occur in response to innocuous agents or allergens, capable of generating an IgE antibody response and are also known as atopic or allergic responses. Type I hypersensitivity occurs in two distinct phases. Sensitization occurs when a genetically susceptible individual is exposed to low doses of allergen transmucosally or transcutaneously. This leads to the activation of T_H2 cells which promote the production of IgE and antigen specific B cells. IgE binds to the high affinity IgE receptor, FcεR1, on the surface of mast cells. Subsequent exposure to the allergen leads to cross-linking of FcεR1 receptors on mast cells with subsequent cell activation and the release of inflammatory mediators.

1.2.1 The ocular immune response

The CD4 co-receptor recognizes conserved regions on MHC class II molecules and defines the T helper cell (T_H) population. T cell activation through the TCR complex and co-receptors CD4 or CD8 requires a second signal to stimulate T cell proliferation. This co-stimulatory signal is initially provided by the molecule B7 (CD80) on antigen presenting cells which ligates CD28 on the T cell surface stimulating the synthesis of the cytokine interleukin (IL)-2. CD4 T cells can further differentiate in two principal directions from their intermediate, post-thymic, T_H0 state, each of which shows marked differences in their pattern of cytokine expression. T_H2 cells are specialized toward activating B cells to produce antibody and thus drive the humoral immune response. This process is initiated by IL-4 which induces transcription factors such as signal transducer and activator of transcription (STAT)-6 [Linehan et al. 1998] which in turn induce and maintains expression of subset-specific transcription factors such as GATA3 [Rao et al. 2000]. T_H1 inflammatory cells preferentially activate macrophages in turn driving the cell-mediated immune response. The differentiation of this cell subtype is initiated by T-bet [Mullen et al. 2001], a T_H1-specific T box transcription factor, with a growth signal subsequently supplied by IL-12 provided by antigen presenting cells, this signal also acts to prolong IFN-γ synthesis. T_H cells show marked differences in the pattern of cytokines (Section 1.2.2.3) which they produce. Both classes secrete IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF). T_H1 cells predominantly secrete interferon (IFN)-γ, IL-2 and TNF-α, whereas T_H2 cells release predominantly IL-4, IL-13, IL-5 and IL-6 [Mosmann et al. 1989]. The compartmentalization of T cell subsets may be important in pathological states where the selection of, e.g., T_H2 cells may lead to sustained IL-4 production resulting in disordered IgE regulation typical of atopy [Parronchi et al. 1991]. *In vivo*, large numbers of T cells have been noted to accumulate in the conjunctiva of patients with chronic allergic conjunctivitis [Allansmith et al. 1979a; Foster et al. 1991]and the preferential accumulation of T_H2 cells in the conjunctiva has been observed in vernal disease [Maggi et al. 1991]. Rodent tissue studies have shown increased numbers of conjunctival T cells following allergen challenge [Carreras et al. 1993] but no human tissue studies exploring T cell population changes during seasonal allergic conjunctivitis have been performed.

Attention has traditionally focused on T lymphocytes as the major source of cytokines. Evidence for mast cells as a potential source for cytokines was first demonstrated by the Abelson murine leukaemia virus (A-MuLV)-transformed tumourigenic mouse mast cell line which constitutively express granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA and to release GM-CSF bioactivity [Chung et al. 1986]. Later, Gordon *et al.*[Gordon et al. 1990a] demonstrated that non-transformed or freshly isolated mouse mast cells in culture constitutively contained TNFα and produced high levels of TNFα on IgE-dependant stimulation. Non-transformed mouse mast cell lines were subsequently shown to secrete a range of cytokines (IL-3, IL-4, IL-5 and IL-6) in response to cross-linkage of FcεR1 [Plaut et al. 1989]. Evidence that human mast cells were a source of cytokines was demonstrated in mast cells from the respiratory tract which were shown to contain both IL-4 and to rapidly release IL-4 bioactivity when stimulated with anti-IgE [Bradding et al. 1992]. Although T cells have been demonstrated to contain mRNA for a number of cytokines, T cell cytokine product has not been demonstrated at multiple tissue locations [Barata et al. 1998; Bradding et al.1992]. Lacking an abundant cytoplasm, T cells may not contain stores of cytokine product, but synthesize these molecules for rapid export. The source of the priming pulse of IL-4 required for T_H2 cell development and subsequent activation to secrete the panel of typical chromosome 5q31 cytokines remains to be determined.

1.2.1.1 Regional immunology of the eye

In 1905, Zirm was credited with the first successful transplant of human cornea [1993]. The success of solid tissue transplantation in the eye, and ability of viruses such as herpes simplex to establish persistent and recurrent infection in the cornea suggested that the expression of MHC molecules at the ocular surface may be restricted. Corneal dendritic cells and macrophages are found at the limbus only [Streilein et al. 1979] but the central cornea lacks cells capable of effective antigen presentation. The cornea is also avascular, limiting the ability of effector immune cells to gain rapid access to this tissue. Compromised host defense is thought to arise from the biological requirement for the cornea to remain transparent, by avoiding the early recruitment of inflammatory cells and their activation.

Antigen presenting cell's within the iris and ciliary body are deficient in their ability to present antigen and activate T cells, even when pretreated with IFN-γ which increased their expression of MHC class II molecules [Streilein et al. 1992]. Under resting conditions the endothelial barrier between the blood and the aqueous humour, the blood-aqueous barrier, and the blood and the retina and vitreous cavity, the blood-retinal barrier, contains tight junctions. These markedly restrict the passage of macromolecules and cells into the aqueous humour, leading to low levels of complement and the acute-phase protein, α-2-macroglobulin, compared with plasma. Aqueous humour contains the cytokines transforming growth factor (TGF)-β and α-melanocyte stimulating hormone. These cytokines have been demonstrated to inhibit T cell proliferation and release of IFN-γ. *In-vivo*, aqueous humour has been demonstrated to suppress delayed type hypersensitivity reactions [Cousins et al. 1991]. The draining of antigens to local lymph nodes is also significantly reduced by the anatomic arrangements within the eye because aqueous outflow occurs predominantly via the trabecular meshwork to the venous circulation leading to splenic rather than lymph node antigen (Ag) presentation. The unique microvascular arrangements, reduced presentation of Ag, preferential Ag presentation away from lymph nodes to the spleen and immunosuppressive nature of aqueous humour have led to the concept of ocular immune privilege [Barker et al. 1977].

1.2.1.2 Conjunctival mucosal immunity

Conjunctival lymphatics drain to local submandibular and cervical lymph nodes. Both cornea and conjunctiva however, form part of the ocular surface where in common with other mucous membranes, the exposed surface allows the direct entry of potential pathogens through the epithelial layer. Like other mucosal surfaces e.g. gut, respiratory and urogenital mucosa, Ag encounters lymphoid tissue in direct contact with the epithelium.

IgA and IgE are thought to play important roles in humoral immunity at mucosal surfaces. IgA is secreted by plasma cells underlying mucosal epithelium in dimeric form. It binds to a secretory component at the basolateral aspect of epithelial cells which enables transfer of the molecule across epithelial surfaces [Solari et al. 1985]. IgA has been demonstrated to play an important role in reducing bacterial adhesion [Mazanec et al. 1993]. IgE is regarded as the key molecule in parasitic defense, particularly toward *Schistosoma sp.* IgE

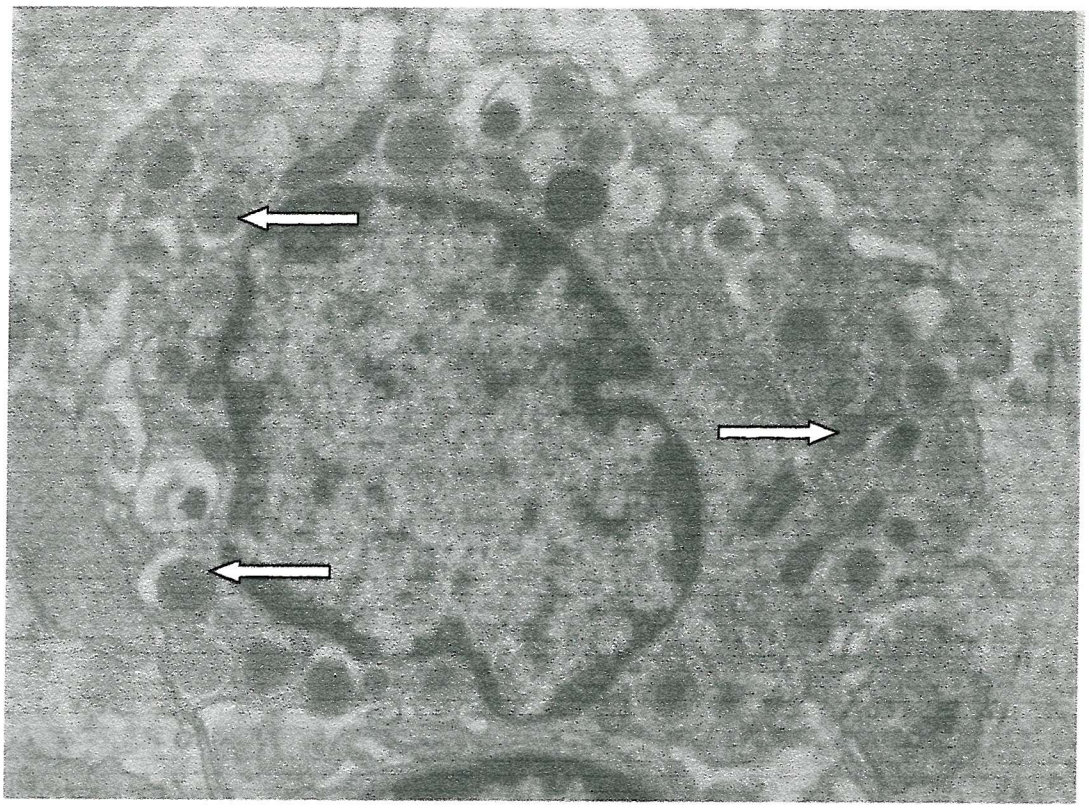
mediated reactions include lacrimation, mucus production, coughing, vomiting and diarrhoea each leading to the expulsion of parasites at their major portals of entry [Sutton et al. 1993]. The principal source of conjunctival IgA and IgE are plasma cells within major and minor lacrimal glands. IgA secreting cells however, have also been immunolocalized, *in-vivo*, to collections of lymphoid tissue beneath the conjunctival epithelium in rabbits [Franklin et al. 1984]. The observation that the generation of IgA at one mucosal surface leads to the secretion of clonally related IgA at other unrelated mucosal surfaces but low titres of circulating IgG, led to the suggestion that mucosal surfaces may be functionally linked . The term mucosal-associated lymphoid tissue or MALT [Wotherspoon et al. 1994] is used to describe this network which comprises bronchial-associated, gut-associated and conjunctival-associated lymphoid (CALT). It is postulated that the homing of the specific immune effector cells to mucosal sites occurs through the expression of addressins unique to mucosal microvasculature [Holzmann et al. 1989]. Lymphocyte migration may be facilitated by upregulation of ICAM-1 on activated endothelium and in HEV's. In turn, the expression of CAM's and activation of endothelium is dependant upon the local cytokine microenvironment, e.g. ICAM-1 expression is powerfully upregulated by TNF-α which may be released from mast cells [Gordon et al. 1991], mucin-like addressin cell adhesion molecule (MAdCAM)-1 [Briskin et al. 1993] guides lymphocyte entry into mucosal lymphoid tissue. The roles of CALT are not yet clearly defined. Tear fluid IgG is known to play important roles in viral neutralization and complement activation leading to viral and bacterial phagocytosis or bacterial lysis. Presentation of ocular antigens may involve conjunctival dendritic cells, Langerhans cells and macrophages. More recently, mast cells have also been shown to participate in antigen presentation [Malaviya et al. 1996].

1.3 Mast cells

An appreciation of the biology of the mast cell is central to the understanding of allergic eye disease. Mast cells display a wide range of functions although their principal physiological roles are incompletely understood. Activation of the mast cell leads to the release of both preformed and newly generated mediators of inflammation [Galli 1993]. This response may be modulated by the location and phenotype of the mast cell, the latter defined by the neutral protease content. The mast cell acts in concert with other

FIGURE 1.3

Transmission electron microscope image of a human lung mast cell



Legend

The abundant cytoplasm contains large granules which display, scroll, grating and lattice patterns (white block arrows) indicating that the mast cell is MC_{TC} phenotype

1.3.1 General characteristics of mast cells

Mast cells were first described by Paul Ehrlich in 1878 [Ehrlich 1878] based upon the metachromatic staining properties of their granules with basic aniline dyes. He used the term *Mastzellen* ('well-fed') because of the presence of abundant granules in the cytoplasm. Although mast cells and basophils share similar staining properties, bear the high affinity IgE receptor, FcεR1, and secrete histamine, mast cells are primarily tissue dwelling cells whilst basophils are in the blood. Mast cells vary in size and shape depending on their location and phenotype. Most human mast cells measure approximately 10μm in diameter, are round with an eccentric monolobed nucleus and are packed with electron dense granules (Figure 1.3) Preformed mediators are held within these granules [Craig et al. 1988]. Granules rich in scrolls are found in human lung mast cells and are thought to reflect a repeating heparin-tryptase subunit. Larger granules consisting of scrolls, gratings and lattices are found in skin mast cells. Here, the granule is more electron dense and overlaid by amorphous material thought to be the neutral proteases chymase and carboxypeptidase (see section 1.2.5).

Mast cells are widely distributed throughout the body and are present at mucosal and serosal surfaces and in lymphoid and connective tissue. They are found in small quantities in bone marrow and lymphoid tissue but unlike basophils (<1.0% of circulating leucocytes) are not found as mature cells within the circulation [Costa et al. 1997]. Mast cells are associated with nerves, blood vessels and tumours, and are often found immediately beneath epithelial surfaces [Galli et al. 1999]. In human conjunctiva, mast cells are found in the substantia propria but not in conjunctival epithelium, although their presence in the epithelium has been noted in chronic allergic conjunctivitis [Allansmith et al.1979a]. The tissue distribution of mast cells places them at potential entry ports for pathogens at mucosal surface, affords them rapid exposure to immune and non-immune stimulation, and places them in close proximity to structures whose function is known to be affected by their mediators. Mast cells play a detrimental role to host tissue in acute allergic reactions. This response however, is likely to have evolved from the protective role of the mast cell in host defense against parasitic infections in which high levels of IgE, mast cell proliferation and mast cell recruitment to areas of parasite load are observed [Galli 1993]. The role of the mast cell in innate immune responses is also supported by

the observations that mast cells may be directly activated by bacterial toxins, haemolysins and lipopolysaccharide [Brody et al. 1998] . Mast cells can process soluble viral and protein antigens and present them to T cells in conjunction with MHC Class I and II molecules *in vitro*, and mast cells have been shown to phagocytose gram -ve bacteria for class I MHC presentation to T cells [Malaviya et al. 1996]. *In vivo*, mast cells have been shown to play an important role in bacterial clearance in murine models of peritonitis through TNFα dependant mechanisms involving neutrophil recruitment [Echtenacher et al. 1996; Malaviya et al.1996]. Mast cells are also likely to play a role in the acquired immune response through their activation and release of preformed and newly synthesized mediators (Section 1.3.4). These products lead to changes in vascular permeability, inflammatory cell recruitment, T cell activation and upregulation of adhesion molecule expression in tissue and inflammatory cells [Galli 1993]. Other roles include the re-modeling of connective tissue and bone, and fibrosis, with increased mast cell numbers observed in areas of chronic inflammation including keloid scars, scleroderma and experimentally induced lung fibrosis [Marone et al. 1997] . Activation of mast cells in these conditions may be different from type I hypersensitivity reactions and a different type of degranulation, termed 'piecemeal', has been observed in inflammatory eyelid skin lesions [Dvorak et al. 1994].

Mast cells have been demonstrated to play important roles in angiogenesis through histamine and heparin secretion [Sorbo et al. 1994] . The effects of mast cell tryptase on endothelial cell proliferation are inconsistent, with a stimulatory effect reported on endothelial cell tube formation derived from foreskin [Blair et al. 1997] , but no effect on human umbilical cord vein endothelial cell (HUVEC) cultures [Compton et al. 1998]. This property may be of considerable importance in tumour angiogenesis and the growth of solid tumours. It is unclear at present whether the accumulation of mast cells around solid tumours represent a host defense response or stimulates the tumour. Uncontrolled mast cell hyperplasia alone may result in a range of disorders known as systemic mastocytosis ranging from relatively benign conditions to mast cell leukaemia [Austen 1992]. The reported effects of mast cells on epithelial cell proliferation, activation of angiotensin converting enzyme, and processing of endothelin and atrial naturitic factor [Brody et al.1998] suggest powerful roles in tissue haemostasis. The association between mast cells and substance P containing neurones in the intestine, and rat mast cell

degranulation and subsequent colonic glucose and water transport suggests a role for these cells in mediating peripheral nervous system homeostasis at tissue level [Bienenstock et al. 1987].

Phenotypic and functional heterogeneity (Section 1.3.3) between mast cells of different species and man may considerably limit the extrapolation of the findings of animal studies to humans. Rodent and human mast cells contain different types and amounts of neutral proteases, the principal component of mast cells by weight. Responses to pharmacological manipulation by agents commonly used to treat human disease e.g. β2-agonists, di-sodium cromoglycate (DSC) and nedocromil sodium also differ markedly [Irani et al. 1989]. Human cord blood mononuclear cells cultured in the presence of IL-6 and stem cell factor gave rise to large numbers of human mast cells. These cells show similar secretory patterns to human lung mast cells (HLMC) when stimulated through the FcεRI receptor [Forsythe et al. 1998] and similar patterns of inhibition of histamine, leukotriene and prostaglandin release when activated through IgE in the presence of a panel of agents used in the treatment of asthma and allergic disorders, including β-adrenoceptor agonists, disodium cromoglycate, phosphodiesterase inhibitors and indomethacin [Shichijo et al. 1998]. Murine mast cell cultures have facilitated the collection of large numbers of cells, overcoming the problems of purification of mast cells from tissue. Using murine cell culture models, mast cells were shown to derive from $CD34^+$ bone marrow precursors, require IL-3 as a critical growth factor and also leave the bone marrow as incompletely differentiated precursors with tissue microenvironment an important factor in final mast cell development (Section 1.3.2) [Rossi et al. 1998]. Human mast cell cultures have recently been developed from cord blood mononuclear cells, bone marrow and fetal liver [Rossi et al.1998]. Important differences between these cultures and murine cell cultures include the apparent lack of a functional IL-3 receptor on human mast cells [Valent et al. 1990]. The unique nature of mast cell development however, requires the addition of growth factors including stem cell factor (SCF) and possibly IL-6 and prostaglandin E_2 [Saito et al. 1996]. As these cells are thought to differentiate in a tissue specific manner, local microenvironmental factors influencing mast cell differentiation in e.g. connective and mucosal tissue are likely to differ. Functional and phenotypic differences between and within mast cell tissue populations are well recognized [Irani et al.1989] and necessitate care in extrapolating results from cell culture to *in vivo* situations. Cadaveric

conjunctiva has recently been described as a source of human mast cells, [Cook et al. 1998] although the pooling and age of donors necessarily limits the study of active pathology. Published studies of tissue changes in conjunctival mast cell populations in allergic disease are limited to tinctorial studies [Allansmith et al. 1978; Allansmith et al.1979a; Tuft et al. 1989a]. The conjunctiva is often further modified by treatment with powerful immunosuppressive agents e.g. topical steroid preparations which may have been discontinued at the time of the study but which have necessarily been used previously to alter the clinical course of the disease .

1.3.2 Mast cell ontogeny and tissue regulation

Mast cells originate in the bone marrow from pluripotent haematopoitic $CD34^{+}$ stem cells. Critical to the development of human mast cells is the pleiotropic cytokine stem cell factor (SCF). This was also named *c-kit* ligand, mast cell growth factor and steel factor and it was successfully cloned by three groups of investigators at about the same time. SCF is the product of the murine steel (*SL*) gene and is produced in two forms. Transcription of the full length SCF mRNA results in a 248 amino acid transmembrane protein which may undergo proteolytic cleavage to form a soluble molecule. Alternative splicing of the SCF mRNA [Flanagan et al. 1991] results in the omission of exon 6 leading to the expression of a 220 amino acid protein which lacks the proteolytic cleavage site and therefore remains on the membrane [Galli et al. 1994a]. Both membrane and soluble form have biological activity [Williams et al. 1990]. The receptor for SCF or *c-kit* (also known as SCFR, CD117) is a member of a family of tyrosine kinase receptors encoded by the *c-kit* proto-oncogene identical with the murine white spotting locus (*W*) [Chabot et al. 1988]. Kitamura *et al.*[Kitamura et al. 1978; Kitamura et al. 1979] examined mice genetically deficient in *c-kit* through a mutation at the white spotting locus (W/W^{v}), mice deficient in SCF through a mutation at the steel locus (SL/SL^{d}), and their congenic (otherwise genetically identical) normal littermates. It was demonstrated that in the presence of any of these defects, mature mast cells could not develop, but that intravenous injection of SL/SL^{d} mouse bone marrow cells into W/W^{v} mice repaired the mast cell deficiency. Unlike other progeny of pluripotent stem cells, mast cells do not develop fully in the bone marrow but leave as morphologically unidentifiable precursors. Work using W/W^{v} mice has demonstrated that basophils are also present in low numbers in the circulation but that

numbers greatly exceed those of mast cell precursors. Morphological studies suggest that these cells are distinct from mast cells and do not appear to represent mast cell precursors [Galli et al. 1994b].

Mast cell survival in tissue is prolonged compared with other tissue and circulating granulocytes. Basophils and eosinophils have a lifespan of days to weeks compared with mast cells which survive for weeks to months [Costa et al.1997]. Stem cell factor is critical to this survival, SCF has been shown to rescue murine mast cells from apoptosis induced by IL--3 withdrawal [Levi-Schaffer et al. 1986], and is necessary for the prolonged survival of human mast cell lines [Valent et al. 1992]. Regulation of mast cell numbers may also be under the primary control of SCF and *c-kit* through a variety of mechanisms. The human mast cell line HMC-1, developed from a patient with systemic mastocytosis, carries a mutation in the gene for *c-kit* leading to constitutive activation of the receptor. Subcutaneous injection of SCF leads to local mast cell hyperplasia in humans [Costa et al. 1996], and increased levels of soluble SCF can be detected in the skin of patients with cutaneous mastocytosis [Nilsson et al. 1996]. *c-kit* and SCF are present in mast cells, but also melanocytes, germ cells, fibroblasts, bone marrow stromal cells and vascular endothelium [Costa et al.1996; Nilsson et al. 1996]. These observations suggest that SCF is probably responsible for the relatively constant numbers of mast cells in tissue under normal conditions but that abnormal expression of SCF or *c-kit* may play important roles in the increased numbers observed in immune reactions, fibrosis and disease [Galli et al. 1992]. Other reported roles for this key cytokine include the enhanced release of preformed mediators from mast cells during IgE stimulation and the stimulation of cytokine generation and release [Bischoff et al. 1992].

Mouse bone marrow-derived mast cells (BMMC) are similar in staining characteristics, size and proteoglycan content to mouse mucosal mast cells (MMC) suggesting that the directed maturation of mast cells by cytokines may take place prior to their appearance in the tissues. Interleukin 3 supports the development of BMMC from progenitors [Levi-Schaffer et al.1986] and IL-4 has been shown to act synergistically with IL-3 in promoting the proliferation of connective tissue mast cells (CTMC) in culture [Tsuji et al. 1990]. Human mast cells however, lack the IL-3 receptor [Valent et al.1990] and neither IL-3 or IL-4 appear to act as growth factors for human mast cells [Valent et al.1992]. Co-culture

of human cord blood mononuclear cells or bone marrow $CD34^+$ cells with 3T3 fibroblasts produces mature mast cells. The growth factor in the culture supernatant was identified as mouse *c-kit* ligand. Further work using long term culture of human BM mononuclear cells with a panel of cytokines supported this earlier work by demonstrating that recombinant human (rh) SCF, but not rhIL-3, rhIL-4 or rhIL-9, was able to induce the differentiation of human mast cells [Valent et al.1992]. Other factors required for the development of human mast cells from cord blood mononuclear cells include IL-6 and PGE_2 [Saito et al.1996]. The different conditions required for the culture of these cells reflects the functional and phenotypic differences between mast cell populations (the limitations of tissue culture experiments have been outlined in Section 1.3.1.). Interestingly, basophils, like murine mast cells have been shown to depend on IL-3 as a specific growth factor [Schwartz et al. 1992]. Human mast cells and basophils therefore appear to depend on two distinct growth factors for differentiation, SCF and IL-3 respectively. Although SCF may be a key regulator of mast cell number, survival and differentiation, these experiments suggest that the local tissue maturation of mast cells may also depend on other cytokines and mediators in the local microenvironment.

The recent development of human mast cell culture may yield information on the cell surface receptors expressed by these cells during their development. Currently, mature human mast cells are typically characterized by their expression of intracellular proteases e.g. tryptase, which are considered unique to this cell [Walls et al. 1990] or by employing the mAb YB5.B8 [Okayama et al. 1995] against the surface receptor, *c-kit*. Other mAb's have been raised against mast cells in skin and tissue culture, but these are incompletely characterized [Hamann et al. 1995]. Alterations in the pattern of cell surface molecules on mast cell progenitors may yield information on cell homing to different locations. Understanding of these mechanisms remains poor, although cutaneous mast cell migration through the expression of $\beta 1$ integrins has been described [Columbo et al. 1995]. Amongst the natural ligands for the integrins include the immunoglobulin superfamily molecules ICAM-1 and VCAM-1, markedly upregulated on activated endothelium, particularly through TNF-α dependent mechanisms. This cytokine has also been shown to have important effects on cutaneous leucocyte recruitment *in vivo* through upregulation of E-selectin [Walsh et al. 1991] and may play an important role in mast cell recruitment, particularly during allergic reactions. SCF has been demonstrated to mediate adhesion

between mast cells and fibronectin [Dastych et al. 1994], and to act as a mast cell chemoattractant [Meininger et al. 1992]. It is likely that SCF plays an important role in regulating local cell numbers not only through cell growth and differentiation but also through chemotaxis. Other cytokines are recognized as chemotactic for mast cells including TGF-β and chemokine RANTES [Nilsson et al.1996], which is also chemotactic for memory CD4 T cells. However, the factors influencing the migration of incompletely differentiated cells to tissue remain poorly understood.

1.3.3. Mast cell proteases

Although proteases form the main secretory product of mast cells by weight [Walls 1995], little is known about their role in human allergic reactions. The three proteases isolated from human mast cells are tryptase [Schwartz et al. 1981] , chymase [Schechter et al. 1986] and carboxypeptidase A [Goldstein et al. 1989]. A cathepsin G-like protease has also been localized to human mast cells [Schechter et al. 1990]. Neutral protease content bestows phenotypic heterogeneity upon mast cells, although the biological significance of this remains incompletely understood. Tryptase is common to all mast cells but present in negligible amounts in basophils, and has not been isolated from other cells. Thus, it is an excellent marker for mast cell activity and assays of tryptase concentration in human tears and plasma can be used to asses the levels of mast cell activation in biological reactions. Chymase, carboxypeptidase and cathepsin G phenotypically distinguishes two distinct sub-types of mast cell. Those mast cells containing all four proteases (MC_{TC}), predominate in connective tissue and skin, whilst those containing tryptase alone (MC_T) prevail at mucosal surfaces and the lung [Irani et al. 1986] although at any given site both types of mast cell are usually present. All of the proteases are stored in a fully active form bound to proteoglycans within the mast cell granules. Tryptase a tetrameric serine endoprotease, forms the majority of the protease content and has several important roles relevant to atopic inflammation. Tryptase has kininogenase activity and the generation of bradykinin increases vascular permeability [Walls et al. 1992]. In animal models, injection of tryptase induces vascular leakage and the accumulation of eosinophils and neutrophils at local sites [He et al. 1997]. The ability of tryptase to cleave at arginine : lysine bonds confers a predicted ability to degrade the small neuropeptides vasointestinal peptide and calcitonin-gene-related peptide which may provide an important role in

neuronal regulation. Fibroblasts and epithelial cells proliferate in response to tryptase. This protease may further upregulate the selective recruitment of eosinophils to areas of inflammation by upregulating ICAM-1 expression and IL-8 release from epithelial cells [Cairns et al. 1996]. Epithelial cell proliferation in response to tryptase might play a role in repair of cationic protein mediated damage secondary to eosinophil activation. This is of particular relevance to corneal injury where these mediators can be demonstrated in corneal ulcers and significantly impair wound healing in chronic atopic eye disease [Trocme et al. 1994a; Trocme et al. 1997]. Mast cells are often located around blood vessels and mast cell-derived tryptase has recently been shown to stimulate sustained IL-8 production in human umbilical vein endothelial cell (HUVEC) cultures [Compton et al.1998]. Tryptase activity appears to depend upon an intact tetrameric structure which in turn depends on the association of the molecule with heparin which has a high negative charge. Conversion of tryptase to inactive monomers is reduced when tryptase remains bound to heparin suggesting that its enzymatic activity is restricted locally to the site of its release.

Chymase is a monomeric endoprotease also bound to heparin within the mast cell granule. It is encoded by genes on chromosome 14 closely linked to the genes coding for cathepsin G. When injected intracutaneously in humans chymase induces a weal and flare response [Hagermark et al. 1972], and produces vascular leakage when injected into rat skin [Seppa 1980]. These actions may synergise with those of histamine, bradykinin and platelet activating factor to produce the marked vascular leakage which characterizes some ocular allergic states. The enzymatic activities of chymase include the conversion of angiotensin I to II, the activation of procollagenase and progelatinase, and activation of IL-1β [Schwartz 1994]. Chymase is released in large complexes with proteoglycan and carboxypeptidease A. The size of the resultant molecule restricts its diffusion and helps to maintain its local activity. The enzymatic activity of chymase is limited by α_1 - antichymotrypsin and α_1 - antitrypsin.

The gene coding for carboxypeptidase A is located on chromosome 3. Carboxypeptidase A is a matrix metalloproteinase bound to proteoglycan complexed with chymase. It's principal role is in peptide regulation through the removal of carboxyterminal residues from polypeptides. The pericellular metalloproteinases play an important role in the

regulation of cytokine receptors and adhesion molecules at the cell surface. Whether carboxypeptidase plays a part in immunoregulation alone, or in concert with other proteases is unknown.

Cathepsin G is found in neutrophils and monocytes and was demonstrated in MC_{TC} mast cells by double immunohistochemistry [Schechter et al.1990]. This serine protease may modulate the conjunctival inflammatory response through its ability to cleave IL-4 and lymphotoxin [Sanderi et al. 1991] and contribute to tissue destruction by degrading elastin [Boudier et al. 1981].

1.3.3 Mast cell heterogeneity

Heterogeneity between rodent mast cell subsets was first described by Enerbeck in 1966 [Enerback 1966b; Enerback 1966a] based upon differences in their staining characteristics and fixation requirements. These populations of cells were later found to differ in the proteoglycans present in their secretory granules. Jejunal mast cells were noted to contain chondroitin sulphate E chains and peritoneal mast cells, heparin chains. The differences in negative charge density between these chains is thought to have led to the observed differences in binding to cationic dyes. Biochemical differences, differences in tissue distribution, size of secretory granules and surface marker expression initially suggested that these cells were not developmentally related. The observations that peritoneal cells could be induced to synthesize chondroitin sulphate E, and that changes in mature mast cell phenotype could be induced when peritoneal and mucosal mast cells were injected into skin, peritoneum and blood of the genetically deficient mast cell mouse *W/W^v* suggested that these cells were derived from common precursors but biochemically heterogeneous [Friend et al. 1998]. These observations supported the hypothesis that mature mast cells might be altered by their local microenvironment. Kitamura [Kitamura et al. 1986] used the term 'transdifferentiation' to describe the observed change from fully

TABLE 1.1

The phenotypic and functional differences between rat and mouse mucosal (MMC) and connective tissue (CTMC) mast cells

PROPERTY	MMC	CTMC
Tissue distribution	rat intestine and bronchial epithelium, mouse bone marrow derived	skin, intestinal submucosa, muscle, serosal surfaces
Mediators		
Histamine content	1.3pg $cell^{-1}$ (rat)	15pg $cell^{-1}$ (rat)
Serotonin	negative	positive
Protease type	RMCP II (rat)	RMCP I (rat)
	MMCP 1 and 2	MMCP 3,4,5,6 and (7)
	tryptase unknown	tryptase positive
	carboxypeptidase A unknown	carboxypeptidase A positive
	chondroitin sulphate E and di-B proteoglycan	heparin proteoglycan
Staining		
Alcian blue/safranin O	blue	red
Berberine sulphate	negative	positive
Newly generated mediators	LTC_4, LTB_4, PGD_2 (rat)	little LTC_4, LTB_4,
	LTC_4, LTB_4, no PGD_2 (mouse)	PGD_2 > MMC PGD_2 (not detected mice)
Surface Forssman lipid marker	negative	positive
Functional responses		
IgE dependant activation	positive	positive
Compound 48/80	negative	positive
DSC inhibition	negative	positive
Theophylline	negative	positive
Growth	IL-3, IL-4, T cell dependant	T cell independent ?

Legend MMCP - mouse mast cell protease

Data based on references [Irani et al.1989; Schwartz 1994]

differentiated cell to precursor and then to a phenotypically different cell based on his adoptive transfer experiments. When reconstituted cells were examined, no granules containing combinations of proteases were observed. Based on these properties, mast cells were divided into two subsets. Mucosal mast cells (MMC) isolated from the small intestine of rats were characterized by the production of chondroitin sulphate E and rat mast cell protease (RMCP) II, relatively low histamine content and similarity to rat mast cells from the bronchial epithelium and murine bone marrow-derived mast cell (BMMC). Connective tissue mast cells (CTMC) were characterized by their content of heparin proteoglycan, RMCP II and their relatively high histamine content. Other differences between these subsets are listed in Table 1.1.

The classification of mast cells into MMC and CTMC extended to humans when the staining characteristics of mast cells from gastrointestinal mucosa were also shown to depend on the fixative used to prepare the specimens. Phenotypic differences were more clearly demonstrated by transmission electron microscopy (Figure 1.3) which was able to demonstrate differences between cells in the same section. The human mast cell subsets are defined by neutral protease content which can be specifically and accurately determined using immunohistochemical methods. The MC_{TC} subgroup have been demonstrated to contain tryptase, chymase, carboxypeptidase A and cathepsin G whereas the MC_T subset contains tryptase alone [Irani et al.1986] . Important biochemical and functional differences exist between these two cell types but also between the same cell type isolated from different tissues. To investigate functional differences in histamine release, mast cells derived from foreskin (~99% MC_{TC}) and lung (~90% MC_T) have been activated by immune and non-immune stimuli. The results of these experiments are summarized in Table 1.2. Functional similarity between human mast cell cultures and HLMC have been discussed (Section 1.3.1) with respect to their response to pharmacological agents. It is of note however, that HLMC's are usually obtained by macerating solid specimens of lung from post-mortem specimens. These cells are likely to differ from those cells obtained by bronchoalveolar lavage in live human subjects which contain mast cells located more superficially and are more likely to be involved in the initial reactions toward inhaled allergen. The differences in response to pharmacological agents has relevance to clinical practice where agents used to suppress mast cell activation may have different activity in different cell populations. Up to 33% inhibition of

TABLE 1.2

Heterogeneity of human mast cells based upon differences in histamine release in response to immune and non-immune stimulation

Activation stimulus	**Lung mast cells (~90% MC_T)**	**Skin mast cell (~99% MC_{TC})**
Anti-IgE	+	+
Calcium ionophore A23187	+	+
Compound 48/80	-	+
Basic polypeptides (e.g. Polylysine)	-	+
C5a (complement protein)	-	+
f-met-peptide	-	+
Inhibitory agents		
Sodium cromoglycate	±	-

Legend

modified from Irani [Irani et al.1989].

histamine release from lung mast cells is obtained using high concentrations of DSC however, it is ineffective at inhibiting histamine release from skin mast cells [Irani et al.1989] (Table 1.2). Differences in histamine release in response to compound such as 48/80, the calcium ionophore A23187 and morphine have also been used to demonstrate functional heterogeneity between human mast cell subsets. By counting the number of chymase and tryptase positive cells within the same sections of human conjunctiva and assuming that these reflected the populations of MC_{TC} and MC_T cells, Baddeley showed that 88% were MC_{TC} and 12% were MC_T in normal subjects [Baddeley et al. 1995]. The low activity of DSC on preventing histamine release from cutaneous mast cells is not reflected by the clinical response to this agent, in allergic conjunctival disease in both periodic and chronic forms of this condition [Juniper et al. 1994]. In the bulbar conjunctiva, a phenotypic shift occurs between the mast cell population in normal subjects which comprises about 88% MC_{TC} and 12% MC_T, to about 48% MC_{TC} and 52% MC_T in subjects with active SAC [Baddeley et al.1995]. Evidence that this change might be influenced by T cells or their released products comes from studies of subjects with disorders affecting T lymphocytes. Patients with acquired immunodeficiency syndrome have a selective deficiency of T helper cells and in gastrointestinal biopsies, where MC_T usually predominant, there is an almost complete lack of MC_T, with normal numbers of MC_{TC} [Irani et al. 1987]. It remains possible that altered expression of proteases may reflect changes in cell maturation. These changes may provide further clues to factors influencing tissue directed mast cell differentiation in man.

1.3.5 Mast cell derived inflammatory mediators

1.3.5.1 Histamine

Mast cell derived histamine is the central mediator of ocular allergic inflammation. Histamine bound to the proteoglycan, heparin, is released on activation of the mast cell and rapidly dissociates from it by cation exchange with extracellular sodium. It is then free to bind to specific cellular histamine-1 (H1), H2 and H3 receptors [Ash et al. 1966] resulting in a wide variety of biological effects (Table 1.3) [Abelson et al.; White 1990].

H1 and H2 receptors have been identified on the ocular surface [Abelson et al. 1981; Weston et al. 1981] . Histamine, directly applied to the conjunctiva leads to vasodilation

TABLE 1.3

The biological effects of H1, H2, combined H1 and H2 and H3 receptor ligation

Histamine receptor class	Biological effect
H1	Contraction of bronchial and gastrointestinal smooth muscle
	Increased intracellular cGMP concentration
H2	Down regulation of T cell mediated cytotoxicity
	Lymphocyte proliferation
	Basophil and mast cell histamine release
	Neutrophil release of lysosymal enzymes
	Down regulation of C2 and C3 production by monocytes
	Increased intracellular cAMP concentration
Combined H1 and H2	Local vasodilation and oedema (secondary to increase vasopermeability) as part of the triple response elicited by intradermal injection of histamine
	Increased eosinophil and neutrophil chemotaxis
	Enhanced expression of C3b receptors on eosinophils augmenting eosinophil mediated damage of *Schistosoma mansoni*
H3	Central and peripheral nervous system neurotransmitter

Legend

Derived from White and Abelson [Abelson et al; White 1990].

and increased vasopermeability, manifest as conjunctival hyperaemia and oedema (chemosis) [Ciprandi et al. 1993a]. Agents such as azelastine which are similar in structure to histamine may bind with similar (or greater) affinity and / or specificity to the receptor but not lead to its activation. Because these agents occupy a site which would otherwise be bound by histamine they reduce the activity of the agonist. They are therefore known as receptor antagonists and can also be used to study the specific biological effects of the agonist. Levocabastine is a powerful H1 receptor antagonist and its topical application to the eye leads to a marked reduction in conjunctival chemosis, hyperaemia and irritation following allergen challenge. This further supports the central role histamine plays in this response [Abelson et al].

The extracellular actions of histamine are regulated by two pathways. Histamine is metabolized by histamine-N-methyltransferase and subsequently by monoamine oxidase to methylimidazole acetic acid. The remainder (about 30%) is metabolized by diamine oxidase to imidazole acetic acid.

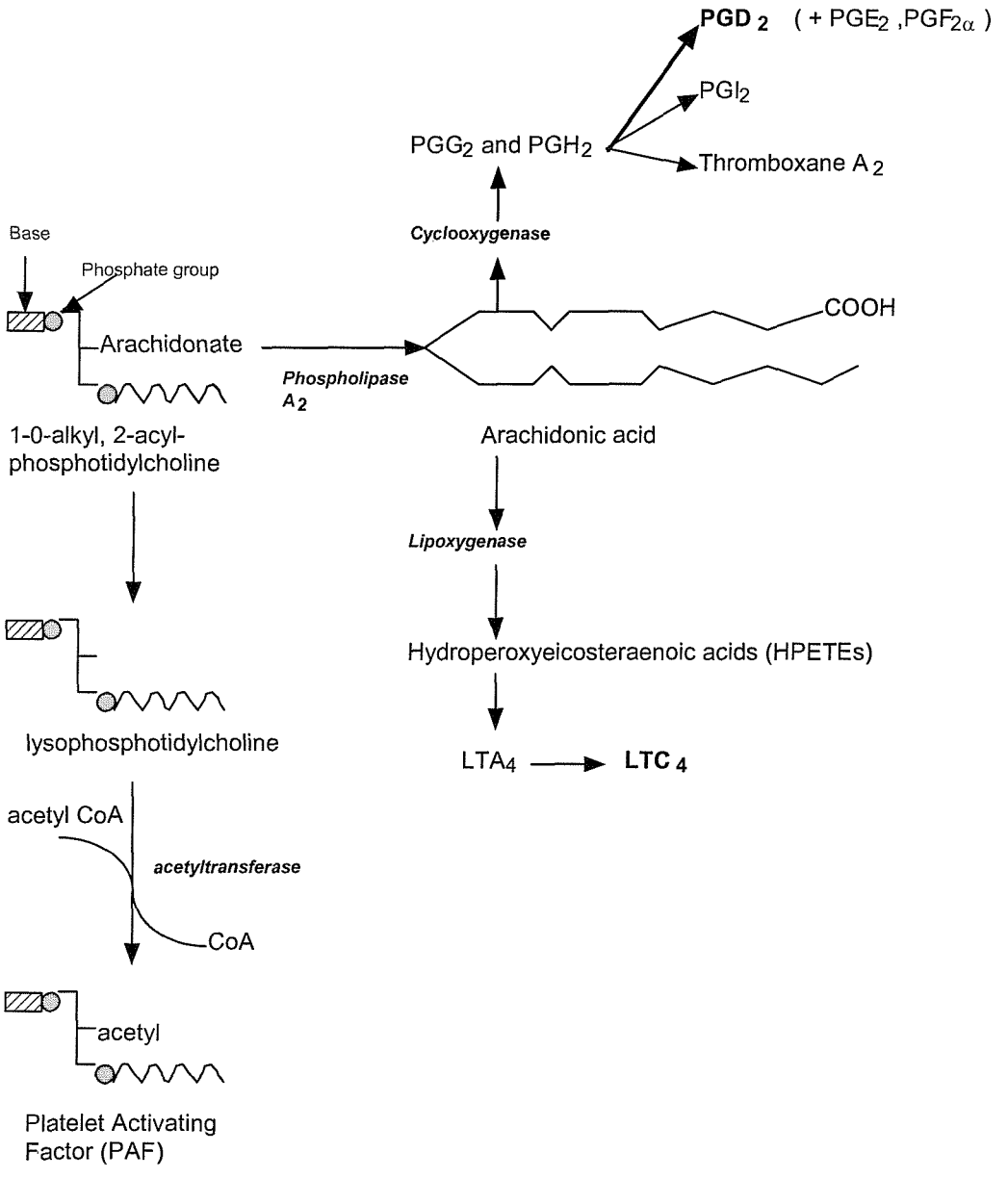
1.3.5.2 Products of arachidonate metabolism

The enzyme phospholipase A_2 liberates arachidonate from cell membrane phospholipids. Arachidonate may then be metabolized by further sets of membrane-associated enzymes (Figure 1.4). The final products of this metabolic pathway are unique for a given cell since each cell type contains a different enzyme repertoire. In human mast cells, the cyclooxygenase (COX) pathway appears to be the principal and rate-limiting route for arachidonate metabolism. The two isoforms of the enzyme COX-1 and COX-2 [O'Neill et al. 1993] are differentially regulated and expressed. COX-1 is constitutively present in most tissues, whereas COX-2 expression is upregulated by cytokines, hormones and growth factors. The gene for COX-2 has been characterized [Appleby et al. 1994] and interestingly its expression has been shown to be downregulated by interleukin-4 [Mehindate et al. 1996]. The COX pathway produces the intermediate endoperoxides prostaglandin G_2 (PGG_2) and PGH_2 which may be further converted to PGD_2 , PGI_2 , PGE_2 , $PGF_{2\alpha}$ and thromboxane A_2.

In human mast cells the major, if not only metabolite produced is PGD_2 [Robinson et al. 1989] (Figure 1.4). The biological effects of this prostaglandin are broad, and include

FIGURE 1.4

Arachidonic acid metabolic pathway in mast cells



Legend:

Bold text Principal metabolites in human mast cells

Italics - enzymes

bronchoconstriction, chemokinesis of neutrophils and inhibition of platelet aggregation [Schwartz et al. 1984]. In addition PGD_2 modulates vascular permeability and when topically applied to the eye leads to acute inflammation [Abelson et al. 1985]. Metabolism of arachidonic acid by lipoxygenase yields the leukotrienes (Figure 1.4) comprising the dihydroxy metabolite leukotriene B_4 (LTB_4) and the sulphido-peptide product LTC_4. Once released LTC_4 is further modified extracellularly to form LTD_4 and LTE_4, which together with the parent molecule LTC_4 were formally known as the slow reacting substance of anaphylaxis, SRS-A. These substances produce contraction of airway smooth muscle and elicit a weal and flare response when injected intradermally in humans. These effects are between 100- and 1000-fold more potent than those produced by histamine, so that although mast cells release significantly smaller amounts of leukotrienes than histamine, their biological activity is comparable [Schwartz et al.1984]. Topical application of LTD_4 and LTC_4 increases microvascular permeability in hamster conjunctiva [Woodward et al. 1985] whilst LTB_4, LTC_4, LTD_4 and LTE_4 are chemotactic for eosinophils [Spada et al. 1986] and neutrophils [Trocme et al. 1989a] in human and rat conjunctiva respectively.

Platelet activating factor (PAF) is a membrane derived phospholipid (Figure 1.4) whose receptor has been cloned [Nakamura M. et al. 1991]. PAF is released from mast cells, basophils, eosinophils, neutrophils and endothelial cells. PAF is reported to be among the most potent of eosinophil chemotactic factors, and its secretion from eosinophils in response to IgE and eosinophil chemotactic factor of anaphylaxis implicates the participation of PAF in eosinophil mediated inflammation. PAF is a potent mediator of vascular leakage, vasoconstriction and platelet aggregation and topical conjunctival application in animals and humans has been shown to reliably reproduce the clinical signs of allergic conjunctivitis [George 1990].

1.3.6 Mast cell activation

The varied methods of mast cell activation are consistent with the pleiotropic nature of these cells and their positioning at sites of potential pathogen entry as well as close proximity to blood vessels and nerves. Activation pathways may be classified into those which are primarily mediated through cross linkage of the high affinity FcεR1 receptor,

termed immune activation, and those which function through other mechanisms e.g. activation via the products of immune reactions, pathogens or physical stimuli. Differences in the mechanisms of mast cell activation may lead to differences in the spectrum of mediator release [Church et al. 1982], degranulation with exocytosis of the granule contents or the stimulation of the synthesis of newly generated mediators or cytokines.

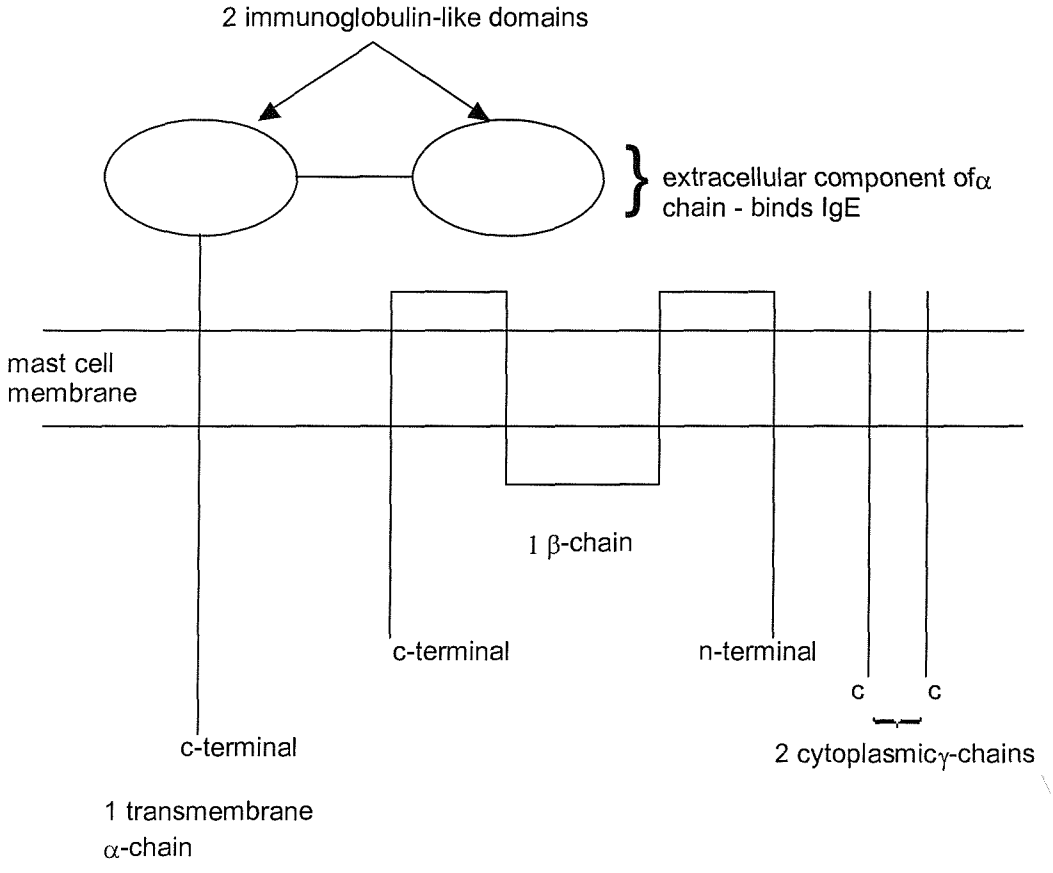
1.3.6.1 Immune mediated activation

Perhaps the biologically most important mechanism of mast cell activation is through the high affinity IgE receptor, FcεR1 (Figure 1.5 and 1.6) present constitutively at high levels on mast cells and basophils, and low levels on eosinophils [Costa et al.1997]. This is the mechanism most relevant to atopic responses and allergic eye disease. FcεR1 binds IgE at a one to one ratio with a high affinity of approximately $10^{10}M^{-1}$ [Kennerly et al. 1993]. It is not the receptor : ligand interaction however, that leads to cell activation, but the approximation of pairs of receptors. These are aggregated through the cross-linkage of surface bound IgE by multivalent allergen (i.e. allergen with multiple antigenic determinants), and relatively few pairs of receptors, approximately 200, are required to cross-link before degranulation is initiated. Whether subsequent conformational changes in the receptor take place following binding is unknown. Transduction of this signal by the receptor is not fully understood, but several mechanisms have been proposed. GTP - binding proteins associated with the intracellular components of the FcεR1 receptor may be activated resulting in dissociation of the molecule and activation or inhibition of other enzyme systems [Bronner et al. 1990; Ishizaka et al. 1978]. Changes in membrane polarity may take place, or activation of a membrane-associated enzyme containing serine within its active site may occur although this enzyme has not yet been characterized. Second messengers important in IgE-mediated mast cell activation include:

i) diacylglycerol, a lipid which may regulate protein phosphorylation and membrane fusion [Kennerly et al. 1979],
ii) cyclic adenosine monophosphate (cAMP) formation by G-protein activation of adenylate cyclase [Ishizaka et al.1978]. This activates protein kinase A (PKA), and

FIGURE 1.5

Schematic diagram of the structure of the high affinity IgE receptor FcεR1

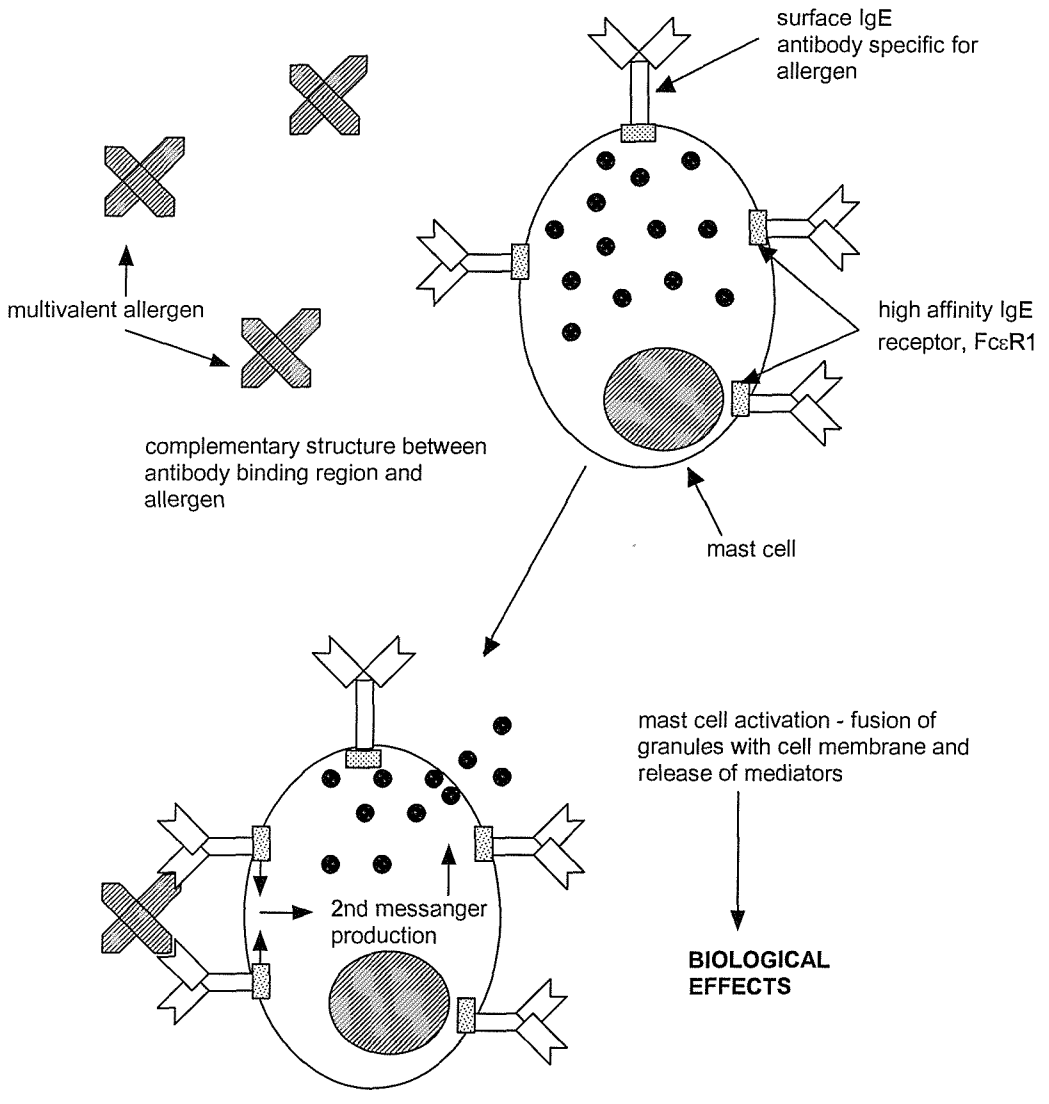


Legend

The high affinity IgE receptor, FcεR1, comprises three associated chains, 1α chain, 1 β chain and two γ chains.

FIGURE 1.6

Mast cell activation through FcεR1 aggregation



allergen binding to IgE leads to physical approximation of Fc εR1 receptors. 2nd messanger generation leads to mast cell activation

Legend

Schematic diagram illustrates the cross-linking of high affinity IgE receptor, FcεR1, to result in release of preformed mediators through cell degranulation

iii) Ca^{2+} mobilized from intracellular calcium stores or possibly through the opening of Ca^{2+} channels in the cell membrane [Penner et al. 1988]. The increase in intracellular Ca^{2+} may lead to activation of PKC, Ca^{2+} binding to the protein calmodulin to form a complex capable of activating Ca^{2+} / calmodulin kinases and possibly membrane depolarization. The final actions of second messengers are to produce a cellular response [Ishizaka et al. 1987]. In the mast cell these may include:

i) fusion between the outer cell membrane and intracellular membranes resulting in exocytosis of stored products [Chock et al. 1985]. Mast cells are able to degranulate in response to repetitive immunological stimuli [Shalit et al. 1993],
ii) biosynthesis of phospholipid derived mediators (see Figure 1.4) or
iii) cytokine gene transcription and generation

Using knockout mice genetically deficient in the ability to make IgE it has been shown that baseline expression of FcεR1 is substantially reduced. Conversely, incubation of human mast cell lines in vitro and murine mast cells in vitro and in vivo with IgE leads to upregulation of FcεR1 [Costa et al.1997]. These observations suggest that a powerful mechanism of positive feedback exists in allergic reactions and host defense against parasites. IL-4 has also been demonstrated to induce FcεR1 in humans [Toru et al. 1996]. This key cytokine in the atopic response may therefore also contribute to the increased production and release of mediators during mast cell activation through IgE-dependant mechanisms.

Other mechanisms of positive feedback may operate to enhance the immune-mediated activation of mast cells. Co-culture of murine fibroblasts and BMMC revealed that transmembrane SCF may activate mast cells through *c-kit*, leading to increased eotaxin and histamine release [Hogaboam et al. 1998]. Both mast cells and fibroblasts are capable of eotaxin release although fibroblasts may be stimulated to produce eotaxin by IL-4 alone. As eotaxin is a potent specific eosinophil chemotactic factor, these interactions represent a potentially powerful mechanism of eosinophil recruitment to areas of atopic inflammation. Eosinophil accumulation is characteristic of chronic forms of conjunctival allergic disease [Abelson et al. 1983a; Trocme et al. 1994a] which are characteristically associated with conjunctival fibrosis. Conditions of primary

conjunctival fibrosis are also associated with eosinophil activation [Heiligenhaus et al. 1998], and the persistent recruitment of leucocytes through sustained mast cell activation may in part be possible through mast cell : fibroblast interaction mediated by SCF.

1.3.6.2 Non-immune mediated activation

Activation of mast cells can occur through the bacterial products, f-met peptide and lipopolysaccharide [Church et al. 1987]. Activation through fibrinogen and fibronectin, complement components and opsonins also support mast cell participation in chronic inflammation and fibrotic processes (Section 1.3.1) whilst activation by the neuropeptide substance P suggests that these cells, closely applied to nervous tissue might mediate between central and peripheral nervous signaling and tissue reactions.

1.3.7 Other cellular constituents of the ocular allergic response

1.3.7.1 Eosinophils

Eosinophils, like mast cells, are primarily tissue dwelling granulocytes and were also described by Paul Ehrlich based upon their staining characteristics. They are distinguished morphologically by the presence of a bi-lobed nucleus and cytoplasmic granules which show a marked affinity for the acidic aniline dye, eosin. The presence of increased numbers of eosinophils in blood and tissues is called eosinophilia. This is characteristic of parasitic infections in which eosinophils are thought to play a protective role, and allergic diseases in which their role is less clear and is often harmful [Weller 1991].

Eosinophil granules may be divided into three types, primary, secondary and tertiary. Primary granules are found in promyelocytes, secondary granules or specific granules, are found in mature eosinophils associated with the third type of granule, the small dense granule. The granule contents are compartmentalized according to structure and function. Secondary granules contain a uniform crystalloid core consisting of major basic protein (MBP) surrounded by a matrix comprising eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN). These proteins have high isoelectric points and are highly cationic. It is this property which is thought to lead to their attraction to the anionic surface of certain parasites. Eosinophils are highly toxic

to certain helminths e.g. *Schistosoma mansoni* and this is thought to reflect their primary biological role in host defense.

Eosinophils are also capable of synthesizing and secreting newly formed mediators of inflammation including LTC_4, PGE_2 and PAF in addition to preformed mediators, whose actions are discussed above (Section 1.2.3) and highly toxic oxygen metabolites including O_2^-, H_2O_2 and $OH^\cdot$. The cytotoxic potential of eosinophil products may therefore be divided into oxygen-dependant (EPO and reactive oxygen species) and oxygen-independent (MBP. ECP and EDN) mechanisms. Cytotoxicity has been clearly demonstrated for mammalian cells, particularly airway epithelium [Venge et al. 1988] further supporting the central role proposed for this cell in the pathogenesis of asthma [Venge et al.1988]. Increased numbers of eosinophils and elevated levels of ECP and MBP have been demonstrated in tears, conjunctival scrapings and conjunctival tissue in all of the major categories of allergic conjunctivitis: SAC, vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC) and contact-lens associated giant papillary conjunctivitis (GPC) [Trocme et al.1994a]. Eotaxin is known to play a central role in the selective recruitment of eosinophils to sites of allergic and non-allergic inflammation [Jose et al. 1994; Ponath et al. 1996]. This chemokine may work synergistically with IL-5, known to be a key regulator of eosinophil growth, survival and release into the circulation from the bone marrow [Collins et al. 1995].

Eosinophils are thought to play a more prominent role in the chronic allergic conjunctivitides, AKC and VKC. They are found in large numbers in the conjunctival substantia propria but also migrate into the epithelium, a site at which they are normally absent [Allansmith et al.1979a; Foster et al.1991]. Their cytotoxic products have been demonstrated in corneal ulcers and also been shown to delay epithelial corneal wound healing *in vitro* and *in vivo* [Trocme et al.1994a; Trocme et al.1997].

1.3.7.2 Neutrophils

Neutrophils comprise the majority (about two-thirds) of leukocytes in peripheral blood and their principal role is the destruction and phagocytosis of micro-organisms. This is achieved through the release of a spectrum of oxidizing chemicals produced by their cell membrane and multiple microbicidal and tissue destructive agents from their granules

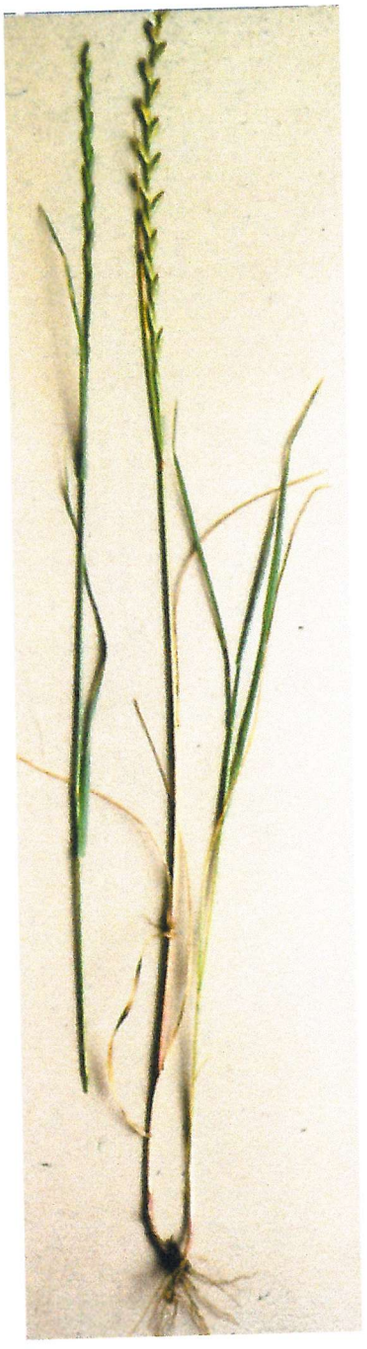
[Gallin 1988]. The toxic effects of neutrophil derived mediators to the host in a range of inflammatory disorders has been well described [Weiss 1989] but the role of the neutrophil in allergic disorders is less clear. Neutrophils have been demonstrated in the normal human conjunctiva by Giemsa staining [Allansmith et al.1978], and neutrophil numbers have been found to be elevated in conjunctival scrapings taken from atopic subjects after high dose topical allergen challenge [Bonini et al. 1988]. Histological studies performed on conjunctiva obtained from patients with AKC and VKC however, failed to demonstrate significant increases in neutrophil numbers [Allansmith et al.1979a; Foster et al.1991].

1.4 Pathogenesis of allergic conjunctivitis

Allergic diseases are thought to affect approximately 20% of the Western population and appear to be increasing worldwide [Strachan et al. 1997] resulting in significant morbidity and mortality. Atopic inflammation of the mucous membrane of the eye in response to airborne allergen is the basis of allergic conjunctivitis, the commonest form of conjunctivitis to present to general practioners in the UK. Mast cell degranulation with the subsequent release of histamine and newly generated products of arachidonate metabolism, LTB_4 and PGD_2, are considered to be central effectors in the inflammatory response but the broad spectrum of acute and chronic disease suggests the involvement of multiple pathological mechanisms which may share disordered IgE regulation as a common pathway. Symptoms of allergic conjunctivitis in himself were first attributed to pollen exposure by Charles Blackley in 1873 [Blackley 1873] and subsequent disease classifications have been based solely upon clinical criteria. A history of atopy and signs of conjunctival injection, oedema and papillary hypertrophy are characteristic presentations of allergic conjunctivitis. The spectrum of disease ranges from mild but unpleasant in SAC in which signs of disease may be minimal or absent, to the less prevalent forms of allergic eye disease, atopic (AKC) and vernal keratoconjunctivitis (VKC) which are often associated with conjunctival fibrosis and corneal epithelial damage. These latter processes may lead to irreversible corneal fibrosis which may ultimately result in blindness.

FIGURE 1.7

Lolium perenne (rye grass)



1.4.1 Pollen

Grass pollen is a common sensitizing allergen in SAC. It is animophilous rather than insect dispersed (entimophilous), with individual grains produced in large quantity and characterized by small size and ease of dispersion [Thompson et al. 1993]. Grasses are probably the commonest plants on earth and those species prevalent in temperate climates of greatest relevance to atopic disease are *Lolium perenne* (Rye grass)(Figure 1.7), *Phleum pratense* (Timothy grass) and *Poa pratensis* (Kentucky blue grass) [Wissenbach et al. 1998]. The principal allergen of rye grass pollen is *Lol p* I, recognized by the large majority of rye grass allergic individuals. The family of group I rye grass allergens however, demonstrate extensive cross-reactivity with other grass pollens and the three dimensional structure of *Lol p* I remains to be determined. The levels of an individual's circulating IgE to rye grass and other allergens can be determined using radioallergosorbent assay (RAST).

Atmospheric levels of pollen vary widely according to season and weather with maximal pollen release during hot, dry springs or summers. Although the level of pollen required to trigger an allergic reaction will vary between susceptible individuals and also within the same person depending on the duration of exposure to allergen within a given season, threshold concentrations of allergen required to generate a response have been estimated as between 10-50 grains m^{-3} [Thompson et al.1993].

1.4.2 Seasonal allergic conjunctivitis

Pollen is the sensitizing allergen in SAC. Produced in large amounts in the summer first by trees, then by grass. Grains of pollen are blown onto the conjunctiva where they dissolve into the tear film. Evidence that SAC is an IgE-mediated disease is provided by a number of studies. Pollen specific IgE has been demonstrated in the tears and sera of affected individuals the levels of which correlate with symptoms of SAC [Hoffmann-Sommergruber et al. 1996]. Transfer of serum from a hypersensitive individual to a non-sensitive individual reproduced the symptoms of SAC in the latter when exposed to pollen [Allansmith et al. 1970]. Histamine and tryptase have been demonstrated in the tears of

patients with active SAC and during the identical symptoms induced by allergen challenge [Abelson et al. 1980; Butrus et al. 1990].

1.4.2.1 Clinical features of SAC

The cardinal symptom of allergic conjunctivitis is itch. Lacrimation is usually minimal and the common association with allergic rhinitis leads to use of the term rhinoconjunctivitis. Exacerbations of symptoms occurs with peak levels of pollen release, locations with high pollen counts and areas of high atmospheric pollution [Varney 1991]. Signs are usually bilateral. The eyelids may be swollen, but marked oedema is atypical and usually reflects eye rubbing. Hyperaemia of the conjunctival vessels leads to a pink appearance. Eversion of the upper eyelid may reveal small elevations of tarsal conjunctiva called papillae formed by oedema around blood vessels restricted in its spread by the tight adherence of the conjunctiva to the underlying tarsal plate (Section 1.1). If excess mucus has been produced, this is present as a thin stringy mass in the lower fornix.

1.4.2.2 Diagnosis of SAC

The diagnosis of SAC is often made on the history alone, as signs may be subtle or absent. Ancillary tests are not usually required although skin prick testing may confirm sensitization to several pollen sub-types [Varney 1991]. Radioallergosorbent tests (RAST) may be used to detect pollen specific IgE in the blood serum. Conjunctival scrapings represent a convenient method to examine the conjunctival cytology for eosinophils although a negative test result is non-contributory to diagnosis [Abelson et al.1983a].

1.4.3 Chronic allergic conjunctivitis

The two principal forms of chronic allergic conjunctivitis are vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). VKC in temperate climates is a condition which affects boys and does not usually persist beyond the age of 14 years [Rice et al. 1971]. AKC however, tends to begin in the teenage years, is closely linked to asthma and atopic dermatitis and frequently results in conjunctival and corneal fibrosis [Tuft et al. 1991]. In both of these conditions, eosinophil derived cationic proteins are implicated in the sight-threatening complications. Although disordered immune regulation and atopy

play an important role in these conditions, several characteristics of the diseases make the study of SAC potentially more rewarding in elucidating the basic pathological mechanisms of allergic eye disease:

AKC and VKC frequently result in treatment with powerful immunomodulatory drugs including topical steroids and cyclosporin A [Foster et al. 1990].
Both conditions are relatively uncommon in the UK, in particular VKC is a condition of dry, hot climates and the disease may be substantially different from that present in UK populations [Tuft et al. 1998]. The clinical course of VKC and AKC in particular are often associated with frequent episodes of coexistent pathology, AKC is frequently associated with bacterial and herpetic infections. In VKC a clearly defined proportion of patients do not display conjunctival hypersensitivity or atopy.

1.4.3.1 Vernal keratoconjunctivitis

Histopathologically VKC is characterized by the presence of increased numbers of mast cells, eosinophils, basophils [Allansmith et al.1979a] and T_H2 lymphocytes [Maggi et al.1991] in the substantia propria. The MC_{TC} phenotype predominate, and a large proportion of these cells have been observed to be degranulated, with both mast cell and eosinophil granules found free in the intercellular matrix [Trocme et al. 1989b]. The localization of mast cells, basophils and eosinophils to a hyperplastic conjunctival epithelium occurs in VKC but not in normal controls . This correlates with the finding of increased levels of IgE, IgG, EMBP, ECP and histamine in the tears [Ballow et al. 1980; Ballow et al. 1983] [Saiga et al. 1991]. The superficial location of these cells can be detected clinically by the presence of eosinophils in conjunctival scrapings . These findings suggest that mast cells, eosinophils and T cells are central effectors in the pathology of VKC. Activated Th-2 cells and mast cells produce a variety of cytokines capable of stimulating mast cell and eosinophil proliferation and degranulation. The transition from an acute to a chronic inflammatory response leads to the deposition of collagen and large amounts of hyaline fibrous tissue to form the characteristic flat topped papillae of the tarsal plate [Lucus 1989]. VKC is sight threatening because corneal inflammation and the formation of a vernal plaque, particularly in the presence of bacterial, viral or fungal infection may lead to irreversible scarring. Initial damage to corneal epithelial cells leads to microerosions which may coalesce to form an ulcer. Later

the deposition of fibrin-like material [Rice et al.1971] can lead to the formation of the vernal plaque. The cationic products of eosinophil degranulation have been implicated as instrumental in this process and in contributing to a second wave of mast cell degranulation prolonging the inflammatory response. EMBP demonstrates a dose-related cytotoxicity for human respiratory and intestinal epithelial cells [Gleich et al. 1979] and delays corneal epithelial wound healing in vitro [Trocme et al.1994a]. ECP has also been shown to be toxic to epithelium [Venge et al.1988] and both EMBP and eosinophil peroxidase stimulate secretion of histamine from rat mast cells [Henderson et al. 1980].

1.4.3.2 Atopic keratoconjunctivitis

Histopathological evidence suggests the contribution of both immediate and delayed type hypersensitivity reactions to the inflammatory process in AKC. Total serum IgE and total and pollen specific tear IgE levels are elevated in AKC when compared with controls but reported symptoms and clinical signs do not correlate with tear IgE concentration [Tuft et al.1991]. The normal conjunctival epithelium is devoid of mast cells, eosinophils, activated T cells ($CD25^{+}$) and T_H2 cells and their presence with increased numbers of antigen-presenting macrophages and dendritic cells [Foster et al.1991] is a striking feature of AKC. The inflammatory cell infiltrate in the substantia propria comprises T_H2 cells, cytotoxic T cells, B cells and large numbers of mast cells [Morgan et al. 1991]. The recruitment of eosinophils to trigger a second wave of mediator release is a disease mechanism common to both AKC and VKC. A major difference however, is the subepithelial fibrosis and resultant conjunctival foreshortening seen in former which is similar to that seen in ocular cicatrizing pemphigoid [Rice et al. 1990] and implicates fibroblast activation. The keratitis produced in AKC is often complicated by concurrent infection particularly with Herpes simplex virus and with time, vascularization of the corneal stroma may result in the deposition of lipid to produce a lipid keratopathy.

1.4.4 Conjunctival allergen challenge (CAC)

A reliable model of allergic conjunctivitis has been produced by the conjunctival allergen challenge studies of Abelson [Abelson et al. 1990] . Since then conjunctival allergen challenge (CAC) has been shown to be a safe and consistent method of reproducing the conjunctival allergic response [Aichane et al. 1993] by triggering IgE mediated mast cell

degranulation. CAC was initially employed to study the acute or early phase response (EPR) to allergen, but it soon became apparent that the conjunctiva exhibited a dose dependant late phase response (LPR) [Bonini et al. 1990]. Although not consistent, the LPR was reproducible using high dose allergen challenge and this appeared to be in keeping with observations of similar responses in other tissues. Studies of the LPR in the skin [Dolovich et al. 1973] and upper [Pelikan 1978] and lower airway [Beasley et al. 1989] strongly suggested that the LPR might provide the link between the IgE mediated, mast cell dependant EPR and clinical disease. A sustained or discrete inflammatory response at these sites following the EPR was typically accompanied by an eosinophil rich cellular infiltration several hours after the initial provocation. In the lung, this was accompanied by the physiological changes of asthma and bronchial hyperreactivity [Montefort et al. 1994]. Investigation of the pathological basis for this response in the eye however, remains in its infancy.

CHAPTER 2

Aims

Mast cells are widely recognized as central effector reactions in allergic diseases through type I hypersensitivity reactions. Mast cell degranulation in human subjects during type I responses has been observed at light and electron microscopic level [Gomez et al. 1986] and 'piecemeal' degranulation observed in rodents provides evidence for histamine secretion *in-vivo* [Dvorak et al.1994]. Mast cells have been shown to accumulate in areas of chronic allergic inflammation in genetically mast cell deficient mice [Galli et al. 1987] and in humans, mast cells accumulation has been demonstrated in a variety of allergic and non-allergic disorders [Austen 1992; Bently et al. 1992; Bienenstock et al.1987; Bradding et al. 1993; Claman 1985; Djukanovic et al. 1990; Galli 1993; Irani et al. 1990a; Schwartz 1994]. Knowledge of the role the mast cell plays in allergic conjunctivitis however, remains in its infancy. Like chronic allergic disease of the respiratory mucosa, the treatment of chronic allergic conjunctivitis e.g. VKC and AKC, involves the use of corticosteroids, powerful immunomodulatory agents which have prolonged and multiple effects on the target tissue. These therapeutic agents limit the conclusions which can be drawn from experimental and clinical studies in these conditions. The accepted, current therapeutic management of SAC however, specifically excludes the use of corticosteroids, making this an ideal condition to study as an *in-vivo* model of allergic disease. In addition, the target tissue, the conjunctiva, is readily accessible for examination using biomicroscopy and small biopsies of tissue can be taken using minimally invasive surgery. These characteristics have been previously utilized by studies using scraping or brush cytology of the tarsal conjunctiva [Abelson et al.1983a; Tsubota et al. 1991] to derive information about tissue changes. The principal limitation of these approaches has remained the recovery of low numbers of inflammatory cells, even when those cells were known to be abundant in biopsy specimens [Abelson et al.1983a]. To improve on the sensitivity of this work, studies by Morgan et al. in 1991[Morgan et al. 1991; Morgan et al.1991] used tarsal conjunctival biopsies and stained the sections with toluidine blue. They demonstrated that mast cell numbers were increased in allergic versus non-allergic subjects. The stained cells were formaldehyde resistant, suggesting that they were mast

cells and not basophils and for the first time, an immunocytochemical technique was employed on conjunctival tissue using the mAb, AA1 [Walls et al.1990], to distinguish mast cells from basophils. Further work showed higher numbers of mast cells in symptomatic patients with AKC than normal controls suggested that mast cells might play a role in chronic allergic conjunctivitis.

Work by Allansmith [Allansmith et al.1978] on conjunctival biopsies calculated cell counts per mm^{-3} in tissue from different conjunctival locations. This work indicated that higher mast cell numbers were found in bulbar than tarsal conjunctiva and corroborated previous work that showed that mast cells and eosinophils were not located in the conjunctival epithelium. This suggested that bulbar conjunctival biopsy would likely yield more accurate information on cell population changes than techniques which studied changes in more superficial cell layers or the precorneal tear film. By employing immunohistochemical techniques on conjunctival biopsies, it is an aim of this work to substantially increase the sensitivity and specificity of detection of granulocytes and lymphocytes and thereby more fully characterize the cell populations in normal conjunctiva and explore changes resulting in allergic conjunctivitis.

Whilst mast cell involvement in allergic reactions through immediate hypersensitivity is well known, the regulation of the immune response through the production and release of cytokines is under the control of T cells. In 1989, Burd [Burd et al. 1989] demonstrated that growth factor dependent and independent mast cell clones could produce a panel of cytokines by IgE and non-IgE stimulation. Mast cells could also be induced to secrete IL-1, IL-4 and IL-6. At the same time Plaut demonstrated the presence of mRNA and protein for the cytokines IL-3, IL-4 and IL-5 in non-transformed murine mast cell lines after cross linkage of the FcεR1 receptor or stimulation with calcium ionophore [Plaut et al.1989]. This work was extended to humans by Bradding et al. [Bradding et al.1992; Bradding et al.1993; Bradding et al. 1995a] who provided evidence that mast cells in respiratory mucosa were a source for IL-4, IL-5, IL-6 and TNFα, and that mast cell cytokine immunolocalization was higher in asthmatic compared with normal airway. Although in this work, IL-8 was not localize to mast cells, but was strongly expressed in the epithelium, later work by Moller [Moller et al. 1993] showed that this important neutrophil chemoattractant was produced by human mast cells. No natural controls were

included in Bradding's studies e.g. asymptomatic asthmatics without treatment, but this work indicated that mast cells might act as an important source of cytokines in allergic airway disease and thus provide a link between bronchiolar hyperreactivity and chronic airways inflammatory disease through the genesis of a late phase response. Atopic individuals were known to show non-specific conjunctival hyperreactivity to hyperosmolar challenge [Ciprandi et al. 1994] and conjunctival allergen challenge had been shown to result in the release of specific mast cell products into the precorneal tear film [Proud et al. 1990]. Although a late phase response in the conjunctiva could be demonstrated by allergen challenge, this was elicited only at the highest doses of allergen and mast cells were not seen in conjunctival scrapings [Bonini et al.1988; Bonini et al. 1989]. This suggested that conjunctival mast cells might provide a link between the early and late phase responses by the production of cytokines, in particular by IL-4. Of particular relevance is the fact that Gauchat had shown in 1993 [Gauchat et al. 1993], that in the presence of IL-4, mast cell and basophil cell lines could induce B cell IgE synthesis through CD40 : CD40 ligand interactions directly, in the absence of T lymphocytes. Pipkorn provided powerful evidence that the study of the natural disease might give markedly differing results to allergen challenge [Pipkorn et al. 1988] and the work in this thesis will encompass the study of conjunctival tissue changes in symptomatic patients with SAC but free of disease modifying treatment, asymptomatic patients, as well as normal, non-atopic controls. In addition, to study the validity of allergen challenge as applied to the conjunctiva, a method known to be consistent and reproducible at clinical level [Abelson et al.1990; Aichane et al.1993; Bonini et al.1988; Bonini et al.1989; Bonini et al.1990; Ciprandi et al.1994] this work will try to develop a safe and reproducible method of allergen specific conjunctival provocation for use in atopic patients sensitive to allergens common to the United Kingdom, and compare the tissue changes elicited by this technique to those observed during 'natural' allergen exposure.

Mast cell numbers, function and activation are known to be closely regulated by the ligand for the *c-kit* tyrosine kinase receptor, stem cell factor [Bischoff et al. 1999; Costa et al.1996; Galli et al.1994a; Irani et al. 1992; Wershil et al. 1992; Zsebo et al. 1990]. Although SCF is known to play a part in mast cell differentiation [Galli et al.1992; Irani et al.1992; Valent et al.1992], mast cells are recognized to be a highly heterogeneous cell population with important differences between mast cell lines, transformed and non-

transformed mast cells in culture, mast cells from different species, mast cells within species but from different tissues and between cell subtypes in the same tissue [Irani et al.1989; Irani et al.1986; Irani et al.1987]. Since mast cell subtypes differ in distribution in different forms of chronic allergic conjunctivitis [Irani et al. 1990b] and T cell clones derived from patients with allergic conjunctivitis show high CD4+ to CD8+ ratios and production of large amounts of IL-4 [Maggi et al.1991], this work will explore differences in mast cell subtypes within conjunctival tissue based on the pattern of cytokine immunoreactivity. In addition, to better understand the biology of this ubiquitous, multifunctional but poorly understood cell, this thesis will seek evidence of autocrine production of SCF by the mast cell under both normal conditions as well as during conditions of active or inactive allergic inflammation.

To summarize, the main aims of this thesis are:

- To define the cellular profile of human conjunctiva in the normal state and compare this to conjunctiva from symptomatic and asymptomatic patients with SAC using sensitive and specific immunohistochemistry.
- To investigate and describe the pattern of cytokines found in conjunctiva from symptomatic and asymptomatic patients with SAC and normal controls, to determine their cellular location and confirm these findings by double staining techniques and the use of *in-situ* hybridization.
- To provide evidence that human mast cells are a source of stem cell factor
- To try to develop a safe and efficacious method of CAC and determine the cellular and cytokine profile of the challenged tissue.

CHAPTER 3

Subjects

Ethical approval for all of these studies was obtained from the Southampton Joint Ethics Committee. In accordance with the tenets of The Declaration of Helsinki, informed, written, consent was sought from each patient prior to participation in all studies after the nature and possible adverse consequences of each study were fully explained.

3.1 Subject recruitment and evaluation

Subjects were recruited to the study from two sources. Patients with SAC and non-atopic subjects to act as controls were invited to contact the Department of Ophthalmology through advertisements placed in the local press and within the hospital. In addition non-atopic control subjects were recruited from patients due to undergo cataract surgery. A detailed history was taken according to a pro-forma. This included a personal and family history of atopy, a general ophthalmic and medical history and a record of the use of any medication.

Inclusion criteria for non-challenge subjects

A history of SAC lasting longer than two years

Positive skin prick to mixed grass pollen.

Exclusion criteria

1. The current use of contact lenses
2. The presence of a pre-existing ophthalmic condition excluding cataract
3. The use of topical ophthalmic medication excluding antihistamines
4. The use of topical antihistamines for a period of two weeks prior to examination
5. The use of systemic anti-inflammatory agents

Examination of the eye was performed using a slit lamp biomicroscope. At low power the overall features of the eyelids, conjunctiva and globe of the eye were examined. Lacrimation was examined at low levels of illumination to avoid increasing reflex lacrimation. A minimal touch technique was employed to reduce artifactual signs to a minimum. Specifically the following features were sought:

lid swelling
conjunctival papillae or follicles
conjunctival hyperaemia
conjunctival oedema (or chemosis)
high lid margin tear strip
signs of other pathology particularly dry eye or non-atopic conjunctival inflammation
The details of the subjects from whom biopsies were taken are given in Table 3.1

A scoring system modified from that devised by Abelson [Abelson et al.1990] was used to score responses to direct questions or findings on examination. This system focused on four main components, itch, lacrimation, hyperaemia and oedema or chemosis (Table 3.2).

TABLE 3.1

Age, sex and symptom score for all subjects from whom bulbar conjunctival biopsies were taken

Group	Biopsy code	Age	Sex	Score	Group	Biopsy code	Age	Sex	Score
Normal	63	63	F	0	OOS CH	38			
Normal	64	90	M	0	OOS CH	39			
Normal	67	71	F	0	OOS CH	40			
Normal	68	78	M	0	OOS CH	42			
Normal	77	81	F		OOS CH	44			
Normal	87	65	F		OOS CH	48			
Normal	88	69	F		OOS CH	49			
Normal	91	72	M	0	OOS CH	54			
Normal	100	51	F	0	OOS CH	55			
Normal	101	91	F	0	OOS CH	71	58	M	
Normal	102	50	F	0	OOS CH	72	34	F	
Normal	103	67	F	0	OOS CH	73	43	M	
Normal	104	77	F	0	OOS CH	126	32	F	
Normal	105	73	F	0	OOS CH	127	60	F	
Normal	106	53	F	0	OOS CH	128	49	F	
Normal	110	82	F	0					
Normal	112	75	M	0	OOS	45	27	M	0
Normal	113	70	M	0	OOS	47	28	F	0
Normal	114	74	M	0	OOS	51	35	F	0
Normal	115	80	F	0	OOS	53	46	M	0
Normal	116	89	F	0	OOS	65	70	M	0
Normal	117	79	M	0	OOS	66	24	M	0
Normal	121	26	F	2	OOS	69	38	F	0
Normal	122	48	F	0	OOS	70	19	M	0
Normal	125	72	M	0	OOS	93	47	F	2
Normal	133	22	F		OOS	95	49	M	0
Normal	134	21	F	0	OOS	96	47	F	0
Normal	135	20	M	0	OOS	98	74	F	0
Normal	136	20	F	0					

Group	Biopsy code	Age	Sex	Score	Group	Biopsy code	Age	Sex	Score
Normal	137	21	M	0	SAC CH 24	107	28	F	
Normal	138	20	M	0		108	59	M	
Normal	139	20	F	0		119	26	F	
Normal	140	20	F	0		120	26	F	
Normal	143	65	F	0		123	36	F	
						124	30	F	
						130	21	M	
SAC	78	48	M	7		141	32	M	
SAC	79	68	F	7		142	45	F	
SAC	80	23	F	7		143	27	M	
SAC	81	28	M	8					
SAC	82	28	M	5		N CH24	111	22	M
SAC	83	72	M	6			118	43	M
SAC	84	58	F	6					
SAC	85	76	M	8		OOS CH 24	147	47	F
SAC CH6	86	44	M			OOS CH 24	148	49	M
	61	7.92	M			OOS CH 24	149	21	F

Legend

N	Normal
SAC	Seasonal allergic conjunctivitis in season
OOS	Seasonal allergic conjunctivitis out of season
OOS CH	SAC allergen challenged out of season with biopsy at 6 hours
OOS CH 24	SAC subject allergen challenged out of season with biopsy at 24 hours
SAC CH 6	SAC subject allergen challenged in season with biopsy at 6 hours
SAC CH 24	SAC subject allergen challenged in season with biopsy at 24 hours

TABLE 3.2

Scoring system to measure the signs and symptoms of allergic conjunctivitis

Conjunctival hyperaemia

0	None
1	Mild
2	Moderate
3	Severe

Chemosis

0	None
1	Mild (detectable using a slit lamp biomicroscope)
2	Moderate (evident without the use of a slit lamp)
3	Severe (ballooning of the conjunctiva)

Lacrimation

0	None
1	Mild (eye feels slightly watery)
2	Moderate (blows nose occasionally)
3	Severe (tears roll down cheek)

Itch (graded by the subject)

0	None
1	Mild (intermittent tickling sensation)
2	Moderate (continual awareness but without the desire to rub the eyes)
3	Severe (continual awareness with the desire to rub the eyes)
4	Incapacitating itch (subject insists on rubbing the eyes)

derived from Abelson et al. [Abelson et al.1990].

3.2 Skin prick testing

As anaphylactic shock is a rare but potentially fatal complication of allergen provocation [Abelson et al.1990], cutaneous and ocular allergen challenge was performed with resuscitation facilities always onsite. One drop of undiluted allergen was placed on the flexor aspect of the forearm and the epidermis was breached by scratching with either a 23 gauge needle or with a standard lancet which indented the epidermis by 1mm. Each site was marked with a ball point pen. A positive control of histamine dihydrochloride and a negative control of saline and albumin diluent were included in each test. 15 minutes was allowed to elapse before the allergen was blotted by tissue paper. The diameter of any weal response was then measured with a millimeter rule, in the case of a response being elliptical, two measurements were made at right angles and an average result taken. A weal diameter of greater or equal to 3mm larger than the negative control response (if any) was taken as a positive reaction in accordance with standard allergy protocols for skin testing [Frew 1992]. The panel of allergens tested for included:

Grass pollen mix (Soluprick ALK Laboratories, Hørsholm, Denmark.)
Tree pollen mix (Soluprick ALK Laboratories, Hørsholm, Denmark.)
Dermatophagoides pteronyssinus (Soluprick ALK Laboratories, Hørsholm, Denmark.)
Cat dander (Soluprick ALK Laboratories, Hørsholm, Denmark.)
Dog dander (Miles Inc, Elkhart, IN, USA)
Aspergillus fumigatus (Miles Inc, Elkhart, IN, USA)

3.3 Conjunctival biopsy

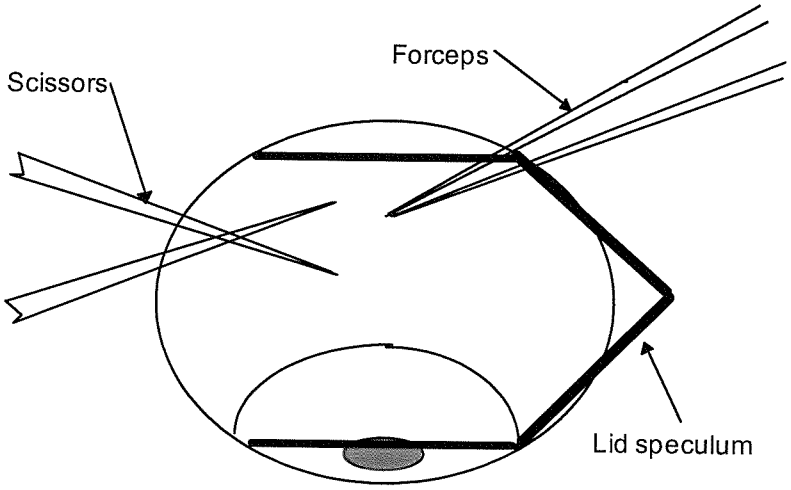
3.3.1 Method

All subjects were examined using the slit lamp biomicroscope immediately prior to conjunctival biopsy. Symptoms and signs were recorded and scored according to the system described above. Topical local anaesthetic drops (oxybuprocaine hydrochloride) were instilled, the patient was asked to lie down and an eyelid speculum inserted to prevent blinking during the biopsy. An area of superolateral conjunctiva was lifted with forceps clear of the underlying Tenons capsule and then dissected with a pair of spring

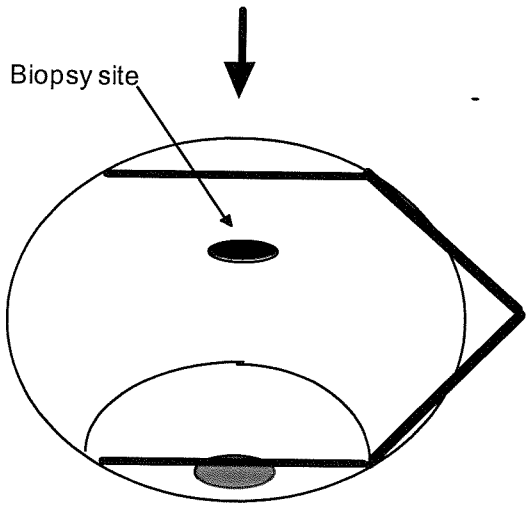
FIGURE 3.1

Diagram to illustrate the method of conjunctival biopsy

A



B



Legend

After instillation of topical anaesthesia the superior bulbar conjunctiva is elevated with forceps and a snip biopsy taken (A) leaving a small defect (B) approximately 2x2mm.

microscissors (Figure 3.1). Topical broad spectrum antibiotic drops (chloramphenicol) were instilled, the speculum removed and the patient asked to continue to use the drops for the next 48 hours. All patients were asked to return immediately or contact the eye unit in the event of an adverse event.

Bulbar conjunctival biopsies were obtained from 80 subjects comprising those with SAC (n=45) and normal controls (n=35) see Table 3.1. Biopsies were taken using local anaesthetic or from patients undergoing regional orbital or general anaesthetic for intra-ocular surgical procedures, most commonly routine planned cataract extraction. The timing of the biopsy was determined by the particular requirements of the study and the predicted pollen count expected for that period based on historical data (Figure 3.2).

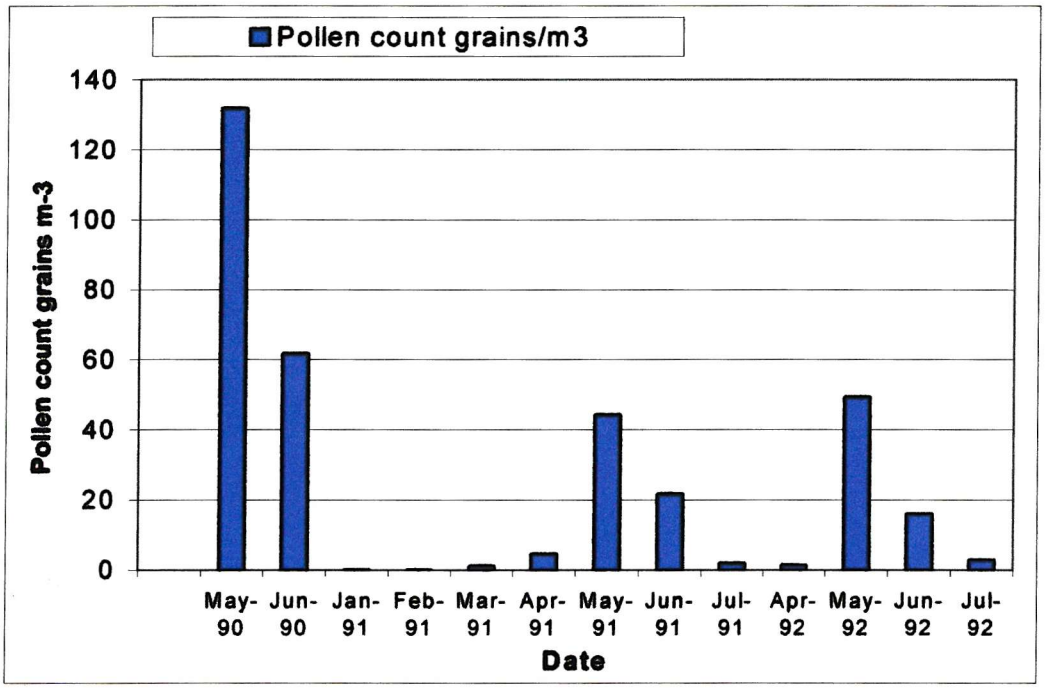
3.4 Pollen Counts

Pollen counts were measured daily by the department of microbiology at St Mary's Hospital on the Isle of Wight. These counts were then used to approximate the counts in Southampton and the surrounding areas from which the subjects were drawn. Pollen counts of approximately 50 m^{-3} have been shown to be correlated with symptoms of allergic rhinitis. The pollen counts applicable to the biopsy times of each of the studies are shown in Figure 3.2. The nature of the climate in the United Kingdom mean that atmospheric pollen levels vary considerably, annually, seasonally, daily and hourly. Sunny, warm summer days are known to result in increased levels of animophilous pollen, rain in particular may reduce pollen levels to zero and depress levels for many hours after the rainfall. This variation in pollen count and the retrospective manner of the data collection from the Isle of Wight had several disadvantages :

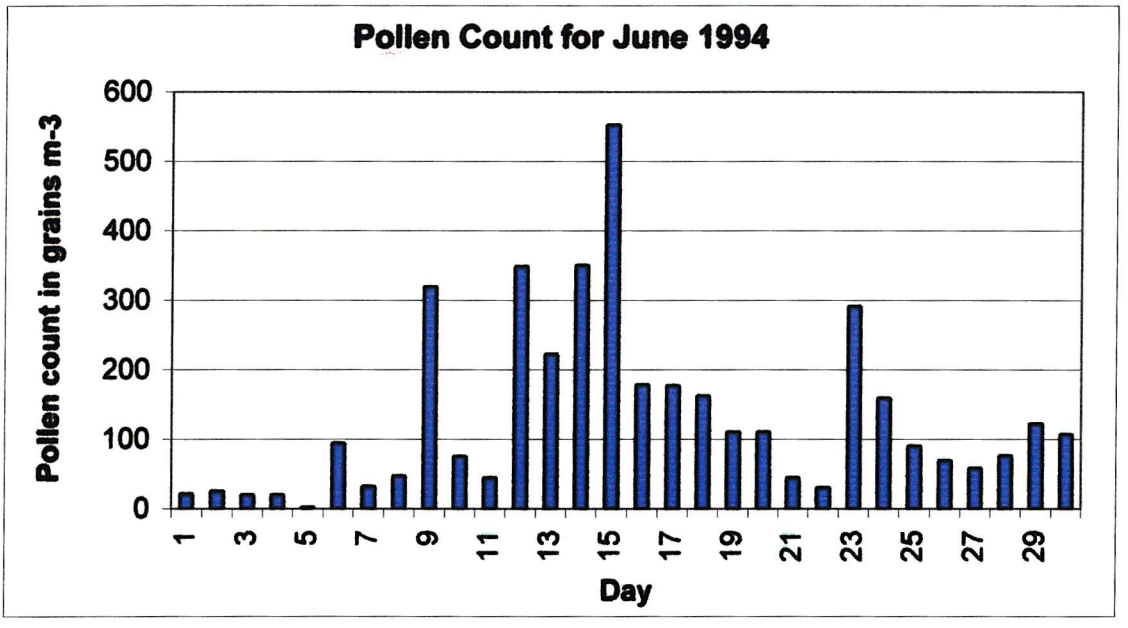
- Within the time span of any given study, the pollen level could not be predicted. For subjects who were called to participate during seasonal studies, or challenged with allergen in season, the baseline symptom score could not be predicted.
- The subjects symptom score could not be correlated to a known pollen level at the time of the study
- Climatic and geographical factors may have led to differences in pollen count on the Isle of Wight and the subjects local environment

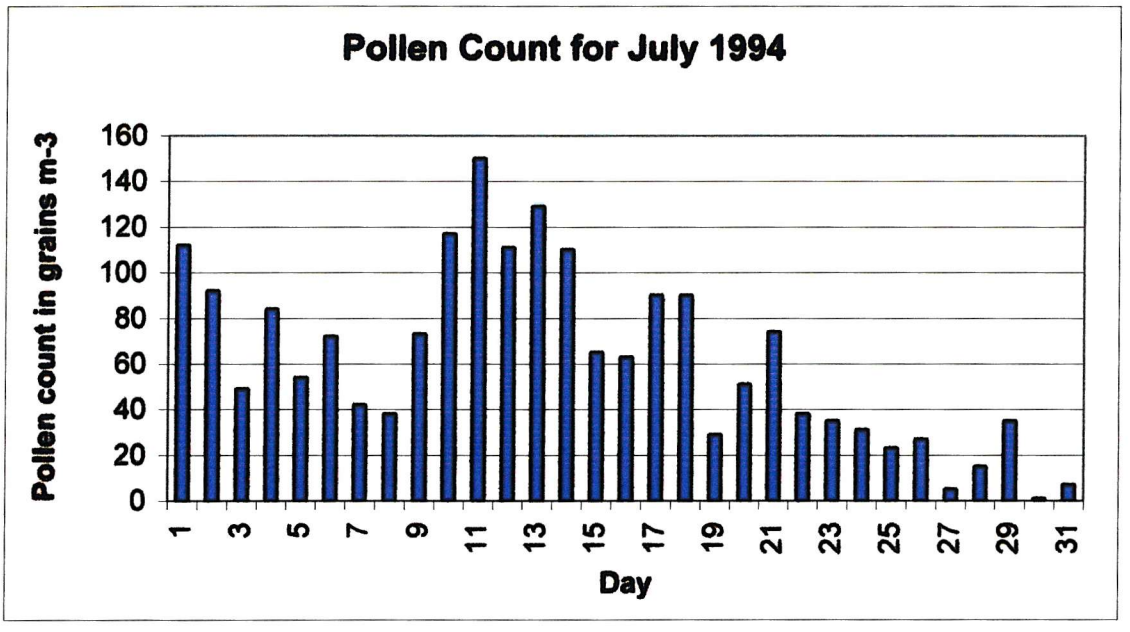
FIGURE 3.2

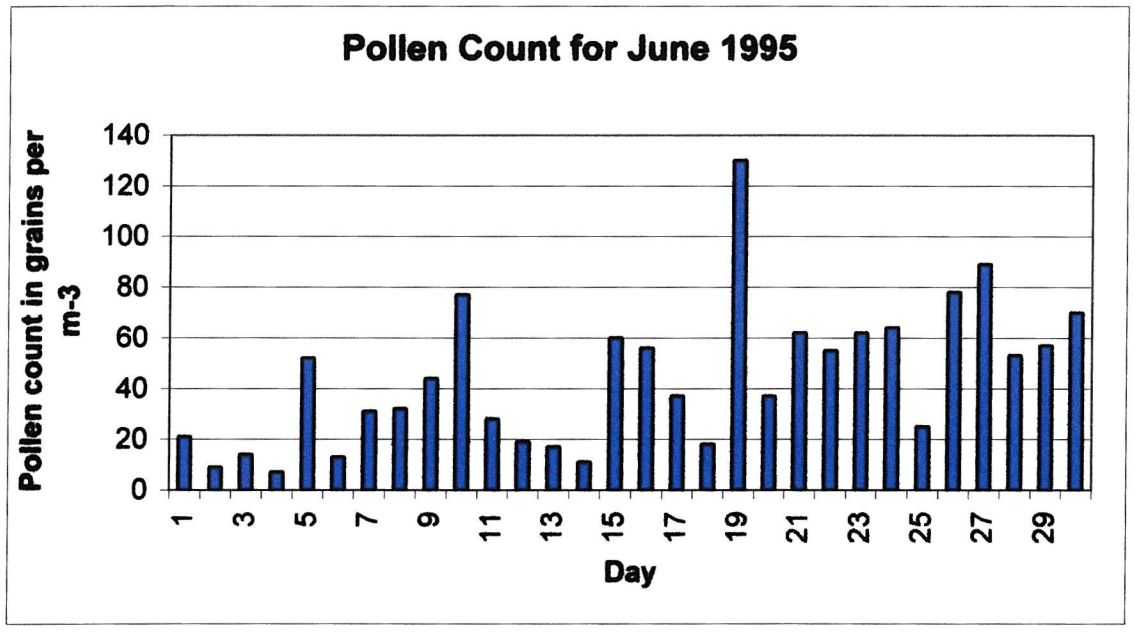
A) Historical variation of pollen counts during summer months

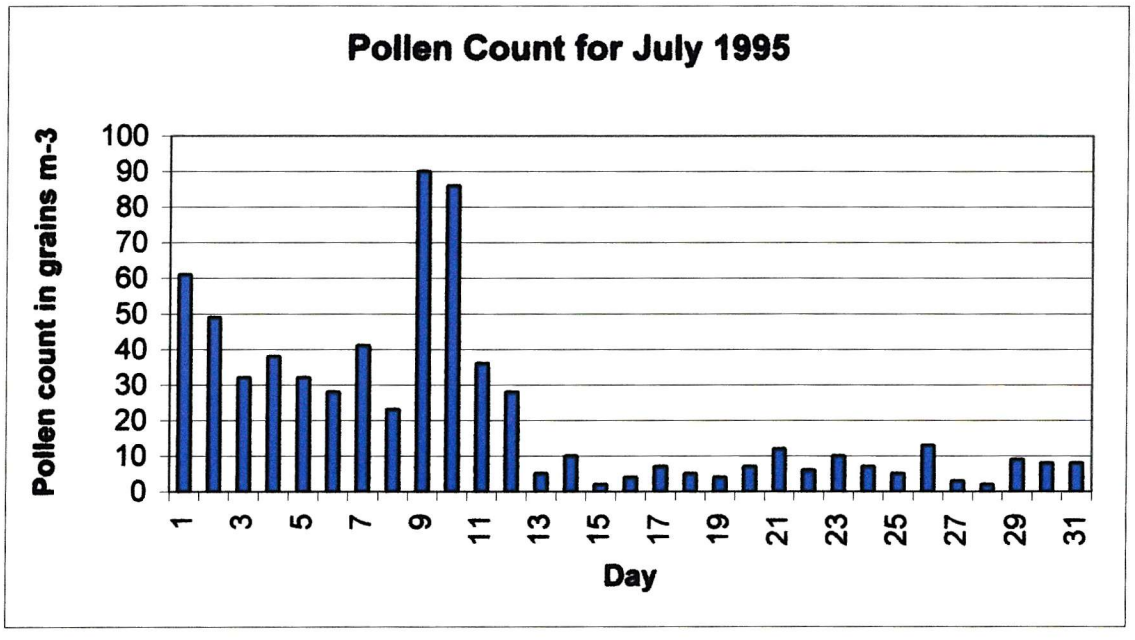


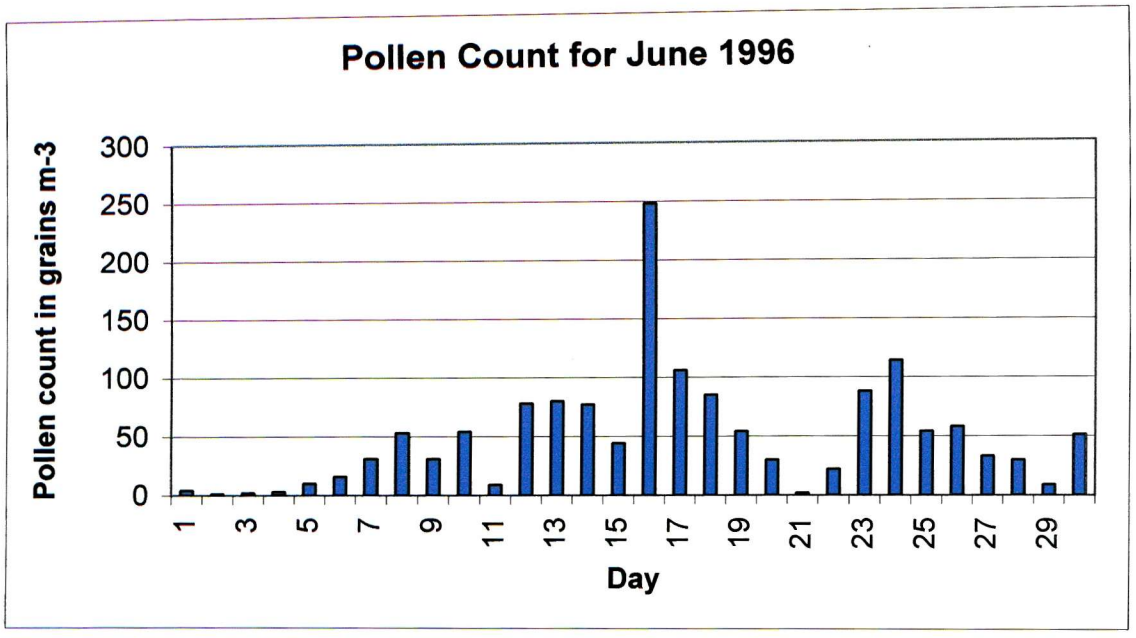
B) Pollen counts during summer months of June and July 1994 when in season biopsies were obtained and comparable months in 1995 and 1996

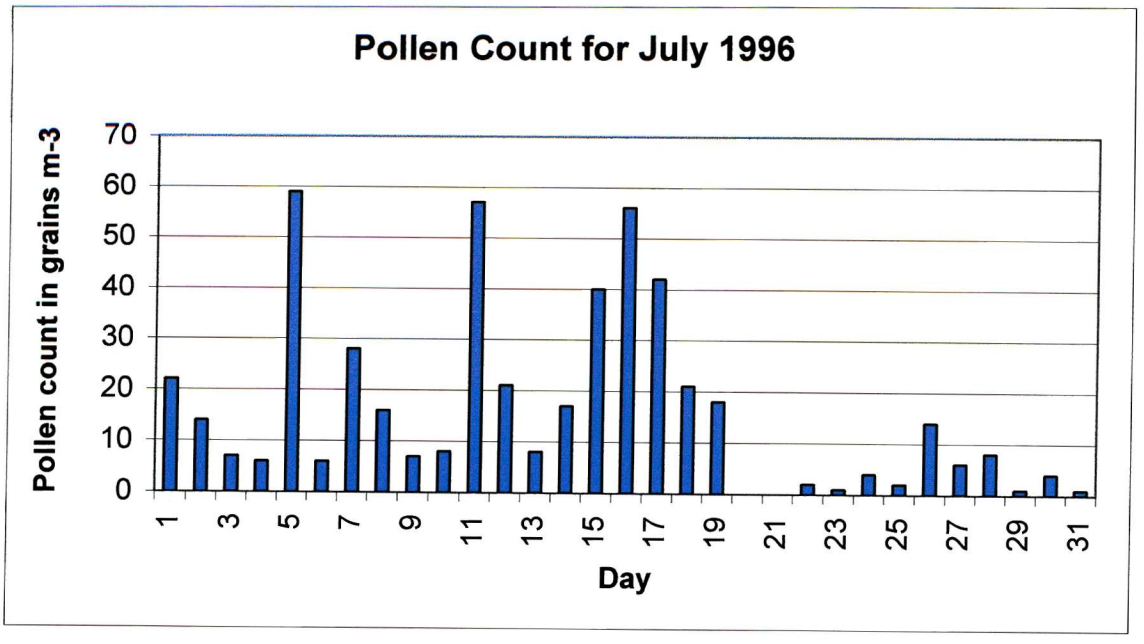





Pollen Count for June 1995
Pollen count in grains per m-3
140
120
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
Day


Pollen Count for July 1995
Pollen count in grains m-3
100
90
80
70
60
50
40
30
20
10
0
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
Day





Legend

Pollen counts obtained from St Mary's Hospital, Newport, Isle of Wight

CHAPTER 4

Materials and Methods

4.1 Introduction

Previous studies examining the pathological changes in the ocular surface in states of ocular allergy have predominantly used data obtained from epithelial cell scraping [Bonini et al.1988; Bonini et al.1989; Canonica et al. 1995; Ciprandi et al. 1993b; Ciprandi et al. 1995a; Tsubota et al.1991] and tear mediator levels [Abelson et al. 1977; Abelson et al.1980; Bonini et al.1989; Fujishima et al. 1995; Tuft et al. 1989b; Uchio et al. 2000]. Previous studies which have examined changes in conjunctival histology employed tinctorial methods of staining to classify and count inflammatory cells [Allansmith et al.1978; Allansmith et al. 1979b; Allansmith et al.1979a]. In this thesis we employed immunohistochemistry to study changes in the populations of inflammatory cells and cells expressing cytokine product and *in-situ* hybridization to seek cytokine mRNA.

4.2 Immunohistochemistry

Immunohistochemistry (IHC), uses labeled antibodies as specific reagents for the localization of tissue constituents, *in situ*, [Coons et al. 1941; Coons et al. 1951]. In order to maximize the sensitivity and specificity of detection, a panel of monoclonal antibodies (mAb) were employed using one mAb subclass, IgG_1 (Table 4.1). Acetone fixation offered the best balance between tissue antigen insolubility and availability for the GMA-embedded semi-thin sections [Britten et al. 1993]. Visualization of the mAb label using enzymatic detection offered a number of advantages over the use of radioactive or fluorescent labeling techniques:

- Safety
- An ordinary transmitted light microscope could be used for detection
- Development of the reaction product took hours rather than days
- The reaction product was stable over a relatively long period of time

- The intensity of the reaction product could be titrated according to the signal to noise ratio

4.2.1 Tissue fixation and processing

Tissue for glycol methacrylate (GMA) processing [Britten et al.1993] was immediately immersed in chilled acetone containing the protease inhibitors phenyl-methyl-sulphonyl fluoride and iodoacetamide (Sigma). The sample was then transferred to a -20°C freezer for overnight fixation. This fixative was replaced by acetone at room temperature (r.t.) for 15 minutes and then methyl benzoate at r.t. for 15 mins. In order to infiltrate the tissue with resin, it was placed in 5% methyl benzoate in JB4 Solution A (GMA solution A, Park Sciences Ltd.) at 4°C for 6 hours. During this period the solution was changed three times. Tissue embedding was achieved by placing the sample in a flat-bottomed Taab capsule (Taab Ltd.) filled with GMA embedding solution (JB4 Solution A, GMA monomer, JB4 Solution B, N,N-dimethylaniline in PEG 400 and benzoyl peroxide). The biopsy was left to polymerize overnight at 4°C and then stored with other biopsies in an airtight container at -20°C with silica resin.

4.2.2 Preparation of slides

GMA embedded biopsies were sectioned on a Leica ultramicrotome at 2μm thickness and floated onto ammonia water (1ml ammonia in 500mls distilled water). After approximately $1^1/_2$ mins the sections were picked up on poly-L-lysine coated slides and allowed to dry at r.t. for between 1 and 3 hours. These slides were either used as soon as dried or stored wrapped back to back in foil at -20°C. In order to retain antigenicity the maximum period of storage was restricted to 1 week. Slides were numbered so that serial sections on successive slides could be identified. In case of loss or damage during processing, two sections were placed on each slide.

IHC was performed using the panel of mouse mAb's directed against specific cellular and cytokine epitopes (Table 4.1). Primary antibodies were applied after tissue peroxidase activity and non-specific antibody binding sites had been blocked by incubating the sections with sodium azide and hydrogen peroxide for 30 minutes and then culture

medium for thirty minutes respectively (see Appendix 1). The antibodies were left on the sections under coverslips to prevent evaporation for between 16-24 hours at r.t. Second stage antibodies labeled with biotin were used to detect the primary antibodies. Streptavidin biotin peroxidase complexes were then used to amplify the signal from the biotin labeled second stage antibodies. Each of these stages was left to incubate for two hours at r.t. The peroxidase complexes were used to develop a chromogen, aminoethylcarbazole (AEC), to give a fine red reaction product.

Between each stage except after incubation in culture medium, the slides were rinsed three times with tris buffered saline (TBS) (see Appendix 1). After each rinse the sections were left immersed in TBS for 5 minutes giving a total time for each wash of 15 minutes. Source of reagents is given in Appendix 1.

4.2.3 Primary antibodies

All mAb's were of the IgG_1 isotype. Primary antibodies were first titrated on GMA embedded conjunctival sections to obtain optimal working dilutions. AA1 was used to label mast cell tryptase, [Walls et al.1990]. MAB1254 was employed to label mast cell chymase. NP57 and EG2 have been shown to label unique epitopes of neutrophils [Pulford et al. 1988] and eosinophils [Tai et al. 1984] respectively. The mAb's 3H4 and 4D9 recognize different epitopes on human IL-4 [Andersson et al. 1990] staining the same cell between 20 - 50% of the time so that by using both mAb's the sensitivity of detection was enhanced. $3H4^+$ cells showed a characteristic ring staining pattern with little cytoplasmic staining whereas $4D9^+$ cells showed cytoplasmic staining. MAB7 to IL5 [McNamee et al. 1991], 104B11 to IL6 , 52B83 to TNFα [Wilkinson et al. 1991] all stain cytoplasmic cytokine. 2D7 to IL8 gives cytoplasmic staining. The IgG_1 monoclonal antibody M9269 was used as an isotype class. The antibody details are given in Table 4.1.

TABLE 4.1

The nature and working dilution of the antibodies used for IHC

Antibody	Raised against human	Working dilution	IgG class	Clone	Source
AA1	tryptase	1 : 200	IgG1	Monoclonal	AF Walls, Southampton University, UK
MAB1254	chymase	1 : 6,000 - 10,000	IgG1	Monoclonal	Chemicon, Harrow, UK.
EG2	eosinophil cationic protein	1 : 200	IgG1	Monoclonal	Pharmacia, Milton Keynes, UK.
NP57	neutrophil	1 : 100	IgG1	Monoclonal	Dako, High Wycombe, UK.
CD3	T cell	1 : 100	IgG1	Monoclonal	Dako, High Wycombe, UK.
3H4	IL4	1 : 600	IgG1	Monoclonal	C Heusser, Ciba-Geigy, Basel, Switzerland
4D9	IL4	1 : 1000	IgG1	Monoclonal	as above
MAB7	IL5	1 : 800	IgG1	Monoclonal	L McNamee, Glaxo, Greenford, UK. (Gift)
104B11	IL6	1 : 30	IgG1	Monoclonal	P Hissey, Glaxo. Greenford, UK.
52B83	TNFα	1 : 50	IgG1	Monoclonal	Celltech, Slough, UK.
2D7	IL8	1 : 2000	IgG1	Monoclonal	I Lindley Sandoz Institute, Vienna, Austria.
MAB 213 to IL13	IL13	1 : 150	IgG1	Monoclonal	R&D Systems Ltd, Oxford, UK.
AB 62-100	SCF	1 : 30	IgG1	Monoclonal	(Biosource / Genzyme, Kent,UK).
M9269	IgG1 isotype control	1 : 30	IgG1	Monoclonal	Sigma, Poole, UK.
Biotinylated anti - mouse $(F(ab')_2$	2^{nd} stage antibody	1 : 300	IgG1	Polyclonal	Dako, High Wycombe, UK.
StreptABC-HRP	3^{rd} stage	1 : 200			Dako, High Wycombe, UK.

Legend: StreptABC-HRP - Streptavidin biotin complexes - horseradish peroxidase

4.2.4 Cell counting

Cells were counted on a Leitz microscope (Leica UK.) using a 1mm graticule and x40 objective to give a total magnification of x400. The whole area of the biopsy was counted systematically by moving the slide the distance of the graticule. Only nucleated cells were counted to avoid counting the same cell twice if it were to enter the plane of the section more than once. The slides were numbered and counted in batches to avoid bias. In order to achieve consistency the slides were checked by a second observer (WRR) and then a proportion recounted after coding by another member of the department. A click counter was used to improve accuracy. The area of the lamina propria (mm-2) and the length of the epithelium (mm) was measured using semi-automated image analysis with Colourvision 1.7.4a software (Improvision, Warwick, UK. And Symantec, Cupertino, CA, USA.) on an Apple Macintosh (Cupertino, CA, USA.) computer (Figure 4.1A and 4.1B).

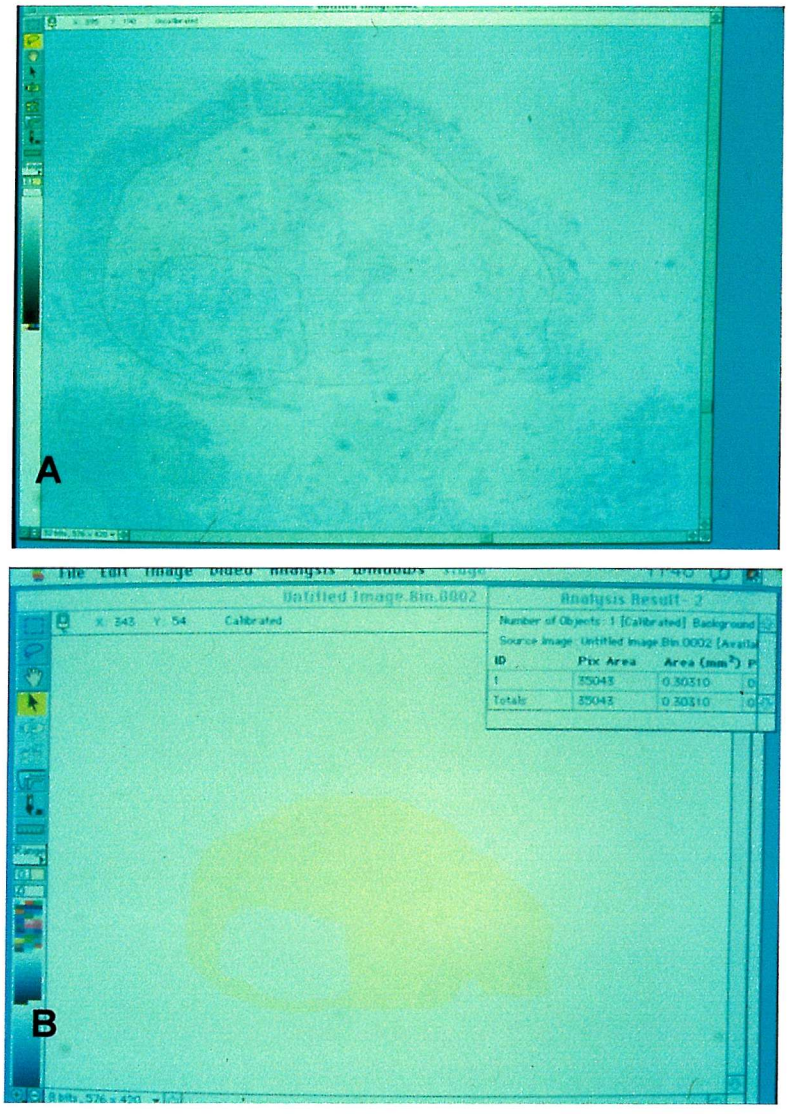
4.2.5 Cytokine co-localization

Serial sections were cut at 2μm allowing several sections to be taken through the same cell depending on its position in the biopsy. In the case of a mast cell, whose average diameter is 10-12μm, there is the potential for up to six adjacent sections through the same cell. The excellent morphology that GMA embedded material provided allows an accurate drawing of the tissue morphology to be made on a clear acetate sheet under high magnification. This was performed by using a camera lucida (Leica, UK). Each cell positive for a given label was circled. The tissue was then drawn on adjacent acetate sheets for sections each 2μm apart, one with cells labeled for tryptase using AA1 and the other for the cytokine of interest. By overlaying the sheets the proportion of mast cells containing a given cytokine, and vice versa, could be calculated. To assess the sensitivity of this technique six serial sections were cut at 2μm and stained with AA1. The proportion of cells co-localizing with the first section was then calculated for each slide.

Using this technique 43% of nucleated mast cells were positive for AA1 in the adjacent section. This represented the maximum sensitivity of the co-localization. In many sections cytokine positive granules were visible in the adjacent section but the nucleus of

FIGURE 4.1

Screen images from Colourvision software to illustrate method of conjunctival biopsy area determination



Legend

A) Freehand tracing of biopsy area with areas for subtraction highlighted. B) The resultant area (shaded yellow) was then calculated in mm^{-2} from the number of pixels

the cell was not so these cells were not counted. This method was therefore adopted when adjacent slides were co-localized. When more than two slides were compared however, increased sensitivity was required. This was necessary when cytokine co-localization was performed in relation to mast cell phenotype studies. For these experiments, three slides were necessary, one labeled for tryptase, the adjacent slide for the target cytokine and the last for chymase (Figure 4.2). To improve the sensitivity of this technique for these experiments, nucleated cells were required to be present for only one of the three sections. Using this modification, over 90% of cells in adjacent sections could be identified.

4.2.6 Area determination

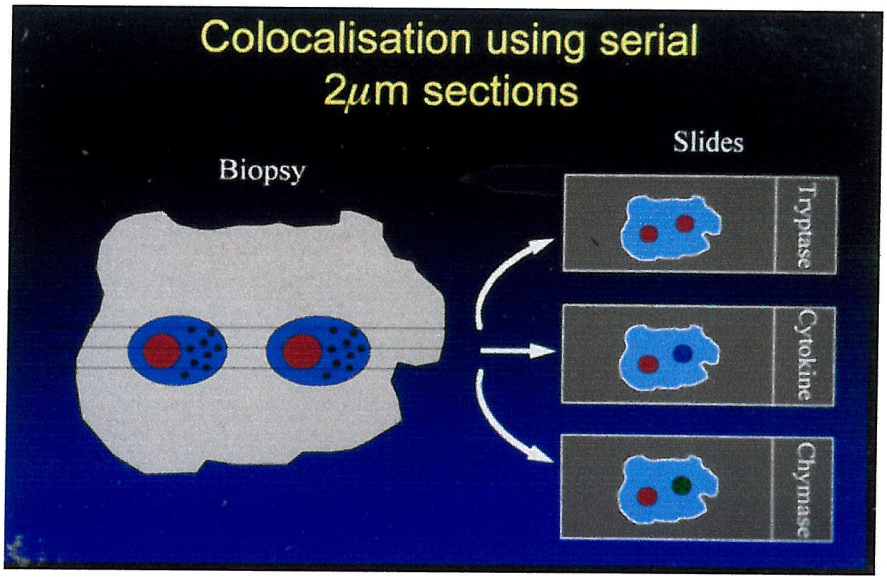
The area of each section was measured using a video interactive display system (VIDS) linked to Colourvision 1.7.4a image analysis software on an Apple Macintosh Computer. This system produced a high resolution image of the slide on a 20' high resolution monitor. Using a mouse, the contours of the section could be traced onto this image. Blood vessels or areas of absent tissue could be mapped and then subtracted, areas of outlying tissue could be added. The system had been calibrated so that the only variable was the power of the objective used. The final area measured in screen pixels was converted into millimeters squared. The length of the epithelium was calculated in the same way to give a length in millimeters.

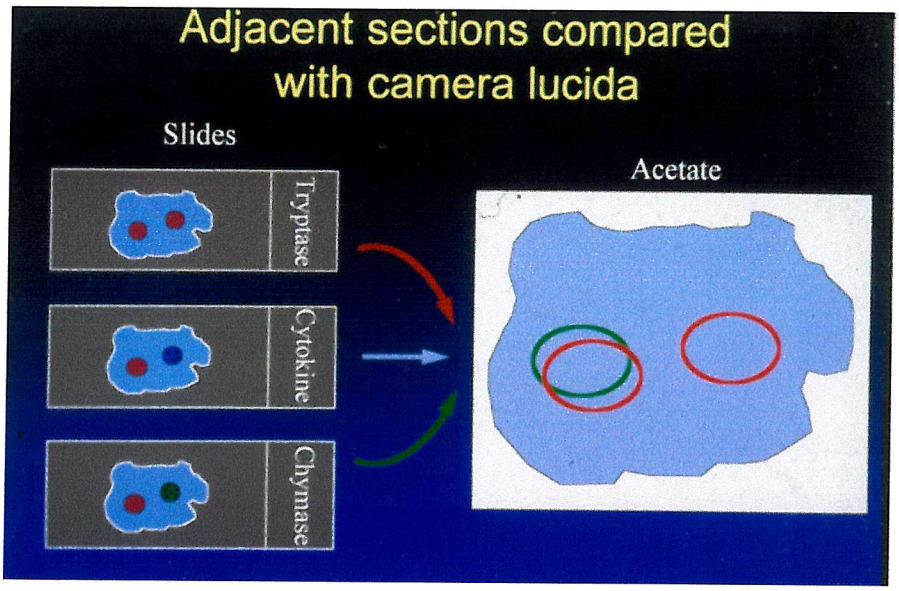
4.2.7 Statistical analysis

The ethical requirement that only one biopsy per patient be performed led to the inability to analyze paired data e.g. biopsy cytokine or cell counts prior to, and following an intervention. The clinical basis of the studies precluded the use of large numbers of individuals, reducing the pooled total cell and cytokine numbers obtained from counts. These counts did not follow a normal distribution when plotted. For the purposes of descriptive statistics, the mean and standard error of the mean were quoted. Non-parametric statistical tests however, were used for statistical comparisons between groups although it was recognized that this would reduce the power of the calculations. Data comparisons between multiple sets was analyzed using the Kruskall Wallis test and

FIGURE 4.2

Diagram to illustrate method of co-localization





between two groups using the Mann-Whitney U test. Spearman's rank correlation coefficient was calculated to examine the correlation between symptom scores and cell counts. Using the method of Bland [Bland et al. 1986], a coefficient of repeatability was calculated for the recounted slides (twice the standard deviation of the log transformed differences in repeated counts).

4.3. Conjunctival double immunohistochemistry

4.3.1 Introduction

This technique was employed to demonstrate both cytokine and lineage-specific cell markers within the same sections. By simultaneously demonstrating both markers at one cellular location, the loss of sensitivity and specificity that entailed from comparisons of serial sections was avoided. Furthermore, by employing fluorescent and non-fluorescent labels and detecting the products by high resolution confocal microscopy using a x 63 oil immersion objective lens, a total magnification of x 630 could be obtained. Because of the decay in fluorescent signal, all slides were kept in the dark at 4°C and read on the same day if possible, but no later than one week following preparation. Images from the confocal microscope were captured using Scanware software (Microsoft, Seattle, USA) and photographed from a high resolution monitor. Tissue was fixed and processed as in section 3.

4.3.2. Materials and Methods

The dilutions of anti-mouse fluoroscein isothiocyanate (FITC)(Sigma) with the primary mAb's listed in Table 4.2, and the nuclear fluorescent label, propidium iodide were first titrated on human conjunctival tissue to determine optimal dilution. IHC was then performed using the monoclonal antibodies listed in Table 4.2. Antibodies were left on the sections overnight (16 to 24 hours) at r.t. after endogenous tissue peroxidase activity and non-specific binding sites had been blocked (see Section 3.4.4). The first stage primary antibodies were labeled with anti-mouse second stage antibodies, bound to either biotin or fluoroscein isothiocyanate (FITC)(Sigma). Streptavidin-biotin alkaline phosphatase complexes (Dako) were used to bind biotin labeled mAb's and develop the

TABLE 4.2

Nature and working dilutions of antibodies used for double IHC

Antibody	**Raised against human**	**Working dilution**	**IgG class**	**Clone**	**Source**
AA1	tryptase	1 : 200	IgG1	Monoclonal	AF Walls, Southampton University
3H4	IL4	1 : 600	IgG1	Monoclonal	C Heusser, Ciba-Geigy.
4D9	IL4	1 : 1000	IgG1	Monoclonal	as above
FITC anti-mouse	mouse	1 : 100	IgG, IgA, IgM		Dako
M9269	IgG1 isotype control	1 : 30	IgG1	Monoclonal	Sigma, UK.
Biotinylated anti - mouse F(ab')2	2nd stage antibody	1 : 300	IgG1	Polyclonal	Dako, UK.
SABC-AP		1 : 200			Dako, UK.

Legend:

SABC-AP, Streptavidin biotin complexes - alkaline phosphatase

TABLE 4.3

The chromogen sequence and control slides used for double IHC

mAb 1	**mAb 2**	**chromogen 1**	**chromogen 2**
3H4	AA1	SABC-AP / fast red	FITC
AA1	3H4	SABC-AP / fast red	FITC
Control slides			
3H4	nil	SABC-AP / fast red	nil
3H4	nil	FITC	nil
AA1	nil	SABC-AP / fast red	nil
AA1	nil	FITC	nil
no antibody		SABC-AP / fast red	
no antibody		FITC	
3H4	nil	SABC-AP / fast red	FITC
AA1	nil	SABC-AP / fast red	FITC

Legend

SABC-AP, Streptavidin biotin complexes - alkaline phosphatase

chromogen, fast red (Appendix 1). This was visualized using visible light or the transmitted light channel of the confocal microscope. The second chromogen, FITC, was detected using a fluorescent channel of the confocal microscope. Propidium iodide was used as a counterstain. Each of these chromogens had a characteristic emission spectrum in either the UV or visible light wavelengths and can therefore be visualized separately, with the images subsequently superimposed. The patient biopsies used to develop this technique were taken from both symptomatic subjects with SAC and controls (Table 3.1). Stringent controls were carried out to ensure that each antibody bound only the chromogen applied first and was not detected by both chromogens. Control slides are listed in Table 4.3 and also included omission of the primary antibody. The sequence for mAb and chromogen used with controls is given in Table 4.3.

4.4 *In-situ* hybridization for conjunctival use

4.4.1 Introduction

The technique of *in-situ* hybridization involves the specific annealing of a labeled nucleic acid probe to a complementary sequence in a fixed tissue. The target sequences chosen for this work were mRNA.

The mRNA target sequences chosen were cross linked and embedded in paraffin blocks limiting their availability for detection. This was overcome by permeabilization and through the choice of probes. Permeabilization, the presence of nucleases and the nature of the tissue, led to tissue and target degradation which limited detection of the target and the eventual quality of the histology. The technique went through a series of refinements to reach a stage where the histology was sufficiently sensitive and specific for quantification.

4.4.2 Materials and methods

4.4.2.1 Probes

Two probes were employed for ISH. To identify mRNA for IL4, a ribonucleic acid probe (riboprobe) was employed. To identify mRNA for stem cell factor two (a cocktail of) oligodeoxyribonucleotide probes (oligoprobes) were used. To achieve high sensitivity for

the detection of IL4 mRNA a single stranded RNA probe was synthesized. The length of the probe was at the upper limit of that required to give a maximal signal. To overcome the relative lack of sensitivity of oligoprobes compared to riboprobes a 'cocktail' of two oligoprobes were chosen.

IL4 riboprobe cloning

A 0.3kb human IL4 complimentary DNA (cDNA) insert from recombinant pUC18 (R & D Systems, Oxford, UK) was excised and subcloned into a vector plasmid pSP70 (Promega, Southampton, UK) in its multiple cloning site containing the promoter sequences SP6 and T7 (For gene and probe sequences see Appendix 3). To separate the IL4 cDNA from the pUC18 plasmid, restriction endonuclease enzymes were used to cleave at specific enzyme recognition sites. HIND III and EcoR1 recognize specific sequences of DNA which flank the cDNA. To prevent re-ligation of the IL4 cDNA to the pUC18 plasmid post cleavage, alkaline phosphatase was used to dephosphorylate the now linearized vector plasmid. T4 DNA ligase (Promega) was then used to incorporate the cDNA into the final recombinant plasmid. Utilizing the principle that new DNA and RNA synthesis proceeds in a 5' to 3' direction i.e. from the 3' end of the template, the orientation of the IL4 cDNA insert was checked. The recombinant plasmid had the following sequence:

SP6 ---- HIND III ---- EcoRV ---------IL4 cDNA--------- EcoR1 ----EcoRV ---- T7

A second restriction endonuclease EcoRV was used to cleave the plasmid at its specific site. If the probe was correctly oriented as above, the resulting fragment:

EcoRV ---------IL4 cDNA--------- EcoR1 ----EcoRV ---- T7

would be ~ 300 base pairs long, if the insert was incorrectly oriented, the resultant fragment:

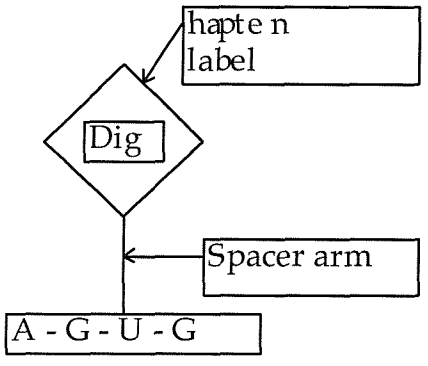
SP6 ---- HIND III -- IL4 cDNA EcoRV --- EcoR1 ----EcoRV ---- T7

fragment a. fragment b.

fragment b., would be ~ 20 base pairs long. The products were run on a 1.2% agarose gel in TAE buffer with markers to determine the molecular weight of the products. (See Appendix 1)

IL4 riboprobe synthesis and labeling

Sense and antisense probes were synthesized and labeled by *in-vitro* transcription of DNA cloned downstream of SP6 or T7 promoter sequences. The label used was digoxigenin (Dig) labeled uridine triphosphate (UTP) (Boehringer Mannheim). During probe synthesis this label is incorporated into the newly synthesized RNA chain such that every 20 - 25th nucleotide would be labeled with Dig-UTP.



To synthesize the antisense probe the IL4 cDNA was cleaved with HIND III and the probe synthesized downstream of a T7 promoter:

SP6 -----HIND III -5'----------mRNA Sense------3'-- EcoR1 -----T7

3' 5'

Antisense probe 5'

5' ---------- EcoR1 -----T7

The sense control probe was likewise synthesized by cleaving with EcoR1 and using an SP6 promoter.

Stem cell factor (SCF) probe synthesis

Cocktails of two antisense and two sense sequence oligonucleotide SCF probes were used. These probes were synthesized commercially (Oswald DNA Services). The sequence of human SCF had been described [Martin et al. 1990] and oligonucleotides chosen which specific for man (Appendix 3, Gene and probe sequences). Each probe was 30 base pairs long and the labeled oligonucleotides were purified from unattached digoxigenin-dUTP using a Sephadex-G 50 (Sigma) spun column procedure. The first antisense probe (P2-Appendix 3) recognized a section of mRNA coding for part of the extracellular domain of the SCF protein. The second antisense probe (P4-Appendix 3) recognized a section of exon 6 mRNA which encoded for the site for proteolytic cleavage. Because a cocktail of these probes was used it was not possible to determine whether either or both of the probes were bound and therefore whether the mast cells expressed the full length SCF mRNA or the alternatively spliced (shorter) mRNA. The advantages of using an oligoprobe cocktail were:

- they were available commercially (and did not therefore need to be cloned)
- they were able to penetrate fixed tissue with greater ease than riboprobes.

The principal disadvantages of using a riboprobe were:

- less label could be incorporated into the probe reducing sensitivity
- the riboprobe-target hybrid was less thermodynamically stable than a riboprobe-target hybrid.

Stem cell factor (SCF) probe labeling

The SCF oligoprobes were 3'- end labeled with digoxigenin -11-dUTP (Boehringer Mannheim, Sussex, UK) at 1:10 molecule ratio by 3'- end tailing procedure using terminal deoxynucleotidyl transferase (tdt) (Promega). To separate labeled from unattached digoxigenin dUTP, a spun column procedure was used. The reagent mixture was poured into an insulin syringe filled with the molecular sieve, Sephadex-G 50 (Sigma) in buffer. This arrangement was then placed into a test tube. The bottom of the syringe was plugged with glass wool to allow the contents of the syringe to pass through it when spun. The column allowed only the larger , labeled probe to escape into the test tube retaining the unlabelled probe.

Checking probe synthesis

To check that the probes had been successfully synthesized, the products were run on a 1.2% agarose gel in TAE buffer.

Checking probe labeling

To check that the dig-labeled UTP had been incorporated into the probes the products were baked onto a nylon membrane at 120°C for 1 hour. Alkaline phosphatase anti-digoxigenin was then used to detect the probe and the colour was developed using the chromogen nitro-blue tetrazolamine 3-bromo 4 chloro I phosphate (NBT - BCIP)(Sigma). The concentration of probe required was determined by titrating different concentrations.

4.4.3 *In-situ* hybridization

4.4.3.1 Preparation of slides

4μm sections were cut on an ultramicrotome and floated onto a water bath at 37°C. They were picked up on aminopropyl-triethoxysilane (APES) coated slides and left in an incubator at 37°C overnight.

4.4.3.2 Tissue fixation and Processing

Each conjunctival biopsy was immersed in 10% neutral buffered formalin for overnight fixation. A cross-linking fixative was chosen to provide greater retention and accessibility of the cellular RNA than a precipitating fixative. The biopsy was then placed in a plastic cassette and processed into molten paraffin wax using an automatic processor (Shandon Hypercentre 2, Cheshire, UK.)(Schedule-Appendix 1).

The major stages in ISH were:

- Permeabilization
- Post treatment fixation
- Hybridization and washing
- Probe detection

Sections were de-waxed by immersion in xylene and then decreasing concentrations of alcohol (100% and then 70%). For all solutions and suppliers see Appendix 1.

Permeabilization

To increase accessibility of the target mRNA the tissue was subjected to mild enzymatic digestion. Sections were washed in diethylpyrocarbonated water (DEPC'd H_2O) followed by immersion in 0.2N HCL for 20 mins at r.t. The sections were then transferred to pre-heated (70 °C) standard sodium citrate (SSC) for 10 mins and washed in DEPC'd H_2O before buffering in 50mM Tris/HCL for 4 mins with a change of solution at 2 mins. Slides were then covered with the enzyme proteinase K (5μg/ml) in 50mM Tris/HCL at 37 °C for 15 mins. Enzyme activity was terminated by washing in pre-cooled DEPC'd H_2O for 20 mins and then pre-cooled PBS for 2 mins.

Post treatment fixation

In order to prevent tissue disintegration and loss of signal after proteinase-K treatment, the sections were re-fixed by immersion in 0.4% paraformaldehyde at 4°C for 20 mins. Sections were then washed in DEPC'd H_2O for 10 mins at r.t.

Hybridization

Prior to the hybridization of probe to target mRNA the sections were incubated in hybridization buffer (Appendix 1) for 2 hours. Fresh hybridization buffer was then added with dig-labeled riboprobe at a final concentration of 5ng/μl at 42°C overnight. The melting temperature (T_m) of the hybrids was influenced by a number of factors:

- Hybridization was RNA to RNA
- The length of the probe (350 b.p.)
- Close homology between the probe and target
- The use of formamide in the buffer which decreased the T_m.

To remove non-specifically bound probe and reduce background, the sections were subjected to stringent washing. The stringency of the washes was increased by using progressively decreasing salt concentrations of SSC (2X for 30 mins, 1X for 15 mins and 0.5X for 15 mins) at 42°C and by adding formamide (30%) to the last wash.

Probe detection

To reduce non-specific antibody binding, slides were washed in 3% BSA/0.1% Triton X100 in TBS for 30 mins. Antidigoxigenin alkaline phosphatase antibody at 1:600 (Sigma) in TBS was then applied for 1.5 hours. Antibody was rinsed off with TBS/0.1% BSA solution 5 mins X2 and then the slides buffered with AP buffer (see Appendix 1) before the chromogen NBT-BCIP (Sigma) was added for 3-6 hours at r.t. in the dark to allow the characteristic blue-black staining pattern to emerge. To reduce background staining levamisole (Sigma) was also added. Slides were then rinsed in tap water prior to mounting in Crystal mount® or used for immunohistochemistry.

Using the SCF cocktail of oligoprobes

The same procedure as above was followed with several modifications:

- Permeabilization was carried out using proteinase K at 5μg ml^{-1} at 37 °C for 20 mins.
- The hybridization buffer differed (see Appendix 1).
- The hybridization temperature was reduced to 37 °C.
- Less stringent washes were employed post-hybridization

Controls

The following controls were used:

Sense probe - This probe had an identical sequence (substituting the DNA-specific nucleotide base uridine for thymidine) to the target mRNA and therefore should not have hybridized with the target.

Positive control - ISH for β-actin was performed using the same procedure as that for ISH for IL-4. For ISH with the SCF oligoprobe cocktail, a probe for the mitochondrial gene C21 was used as a positive control.

RN-ase pre-treated tissue - Tissues were treated with RN-ase (RNase A, Sigma, Poole, UK) at 150μg ml^{-1} for 30 mins prior to hybridization at 37°C. This step was incorporated to ensure that the target nucleic acid was in fact RNA.

Separate and simultaneous detection - To control specifically for dual ISH / IHC both techniques were performed separately on adjacent sections from the same conjunctival biopsy and different biopsies and consistency sought between these results and those obtained from double staining.

4.4.4 Cell counting

Formalin fixation and proteinase K digestion led to loss of tissue morphology and increased tissue separation. For these reasons cell counts were expressed as a percentage of the positive cells in that section and absolute areas were not measured. Cell counts were performed without knowledge of the tissue source on a Leitz microscope (Leica, UK) using a 1mm graticule and x40 objective to give a total magnification of x400. The whole area of the biopsy was counted systematically.

4.5 Sequential *in-situ* hybridization and immunohistochemistry

Sequential ISH/IHC was performed to overcome difficulties in distinguishing SCF $mRNA^+$ and $AA1^+$ cells from SCF $mRNA^-$ and $AA1^+$ cells. This problem arose because the signal obtained using the SCF cocktail of oligoprobes was strong enough to distinguish from background staining but obscured by the strong granular staining obtained with AA1. Sequential sections were cut at 4μm thickness and adjacent sections processed for ISH using the cocktail of oligo probes described above (and Appendix 1) or immunostained using the mAb's AA1 to mast cell tryptase and MAB1254 to mast cell chymase. Sections were then examined using a camera lucida (Leica UK Ltd.).

4.6 RT-PCR for SCF mRNA in isolated human mast cells

Total cellular RNA was extracted from mast cells obtained from purified foreskin tissue using a guanidium-isothiocyanate / acid phenol extraction procedure (Promega, Southampton, U.K.). mRNA's were converted into cDNA by reverse transcription using AMV reverse transcriptase (10U)(Promega) and oligo dT_{15} (1μg) as primer for 1 hour at 42°C. One microlitre of the cDNA was then used as the template in a 25μl polymerase

chain reaction (PCR) containing 1 unit of Taq DNA polymerase (Promega), 200μm of each primer, and 3mM $MgCl_2$ in the reaction buffer. Two sets of primers aligned at 61-90 and 342-371 in the human SCF mRNA sequence (see Table A1, Appendix 3 and reference [Martin et al.1990]) flanking a 311 base pair (bp) fragment which is within the coding region for the SCF extracellular domain and the second set of primers aligned at 342-371 and 742-771 positions flanking a 430 bp fragment which is within the exon 6 encoding the proteolytic cleavage site of SCF. The reaction mixture was amplified by 30 cycles of PCR, each consisting of denaturation at 94°C for 1 min and annealing / extension by electrophoresis on a 2% agarose gel, followed by ethidium bromide staining, and visualized under UV illumination. The PCR without the cDNA template was performed under the same conditions as a negative control. PCR for the constitutive transcript adenine phosphoribosyl transferase (APRT) was performed using the same procedure with specific primers for APRT as a positive control.

CHAPTER 5

Cellular changes in SAC

5.1 Introduction

Seasonal allergic conjunctivitis is the commonest ocular allergic disease. Although not sight threatening, symptoms of itch, lacrimation and mucus discharge may be extremely irritating and are a frequent source of patient self-referral to general practitioners. Exacerbations of symptoms coincides with peaks of pollen release and in areas of high atmospheric pollution [Varney 1991], and symptoms remit when the pollen count falls.

Mast cell activation is thought to be central to the genesis of this acute allergic response. High affinity FcεR1 receptors are aggregated by multivalent allergen cross-linking cell surface IgE. This leads to cell activation and the release of histamine and newly synthesized lipid mediators including prostaglandin D_2 and the sulphidopeptide leukotriene (LT), LTC_4. Topical application of several of these agents reproduces the signs of allergic conjunctivitis [Ciprandi et al. 1993a; Woodward et al. 1985] and histamine, leukotrienes, and the unique mast cell neutral protease, tryptase have been demonstrated in the tears of atopic individuals following allergen challenge [Bisgaard et al. 1985; Butrus et al. 1990; Proud et al. 1990]. Mast cell release of histamine and leukotrienes may also contribute to inflammation by recruitment of eosinophils and neutrophils [Woodward et al. 1986] and more recently, mast cells have also been shown to influence the allergic inflammatory response by the secretion of a range of cytokines [Gordon et al. 1990b]including interleukin-4 (IL4) [Bradding et al.1992] and TNFα [Gordon et al.1990a]. This capacity to rapidly release cytokines, which can occur prior to T cell ingress, confers upon the mast cell the ability to orchestrate the immune response as well as participation in mediating the acute allergic response.

Evidence provided by tear assays suggests that there is local IgE production in the conjunctiva. Pollen specific IgE has been demonstrated in the tears and sera of affected individuals with a highly significant correlation between the former with symptoms of SAC [Hoffmann-Sommergruber et al.1996]. The observation that IgE production by B cells may be induced by mast cells independently of T cell help [Gauchat et al.1993] suggests that mast cells may be involved in the autoregulation of IgE production through CD40 / CD40 ligand dependent mechanisms in the presence of IL4, independent of T cell activation.

Allergic diseases at other mucosal sites are now recognized to occur in association with a characteristic pattern of granulocyte-rich inflammation, involving neutrophils in acute responses and a striking predominance of eosinophils in the chronic disease state [Djukanovic et al. 1990]. The role of the neutrophil in allergic conjunctivitis is however, unclear. Neutrophils but not eosinophils have been demonstrated in the normal human conjunctiva by Giemsa staining [Allansmith et al. 1978], and neutrophil numbers have been found to be elevated in conjunctival scrapings taken from atopic subjects after high dose topical allergen challenge [Bonini et al. 1988]. Histological studies performed on conjunctiva obtained from patients with the chronic allergic conjunctivitides, atopic (AKC) and vernal (VKC) keratoconjunctivitis however, fail to demonstrate significant increases in neutrophil numbers [Allansmith et al. 1979a; Foster et al. 1991].

The presence of large numbers of eosinophils in the lamina propria is characteristic of AKC and VKC [Allansmith et al. 1979a; Foster et al. 1991] as is the migration of these cells into the epithelium. The presence of eosinophil granule major basic protein (MBP), Charcot-Leydon crystal protein [Udell et al. 1981] and eosinophil cationic protein (ECP) in the tears of patients with VKC [Leonardi et al. 1995] also strongly suggests eosinophil recruitment and subsequent activation in these conditions. Eosinophils have been demonstrated in conjunctival scrapings taken from patients with symptomatic SAC but may be absent in some patients [Abelson et al. 1983a]. The failure to recover eosinophils from conjunctival scrapings should not preclude the diagnosis of SAC.

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

5.2 Materials and Methods

5.2.1 Subjects

The scoring system devised by Abelson et al. [Abelson et al. 1990] was used to evaluate all the subjects recruited. All patients refrained from use of systemic or topical medication for at least two weeks prior to their entry into the study. All patients were free from other ocular disease and no patient wore contact lenses. Conjunctival biopsies were obtained from 8 atopic patients with SAC in season, (5 male, 3 female, mean age 50.1 years, range 23-76) 12 atopic patients with SAC out of season, (6 male, 6 female, mean age 42 years, range 19-74) and 20 non-atopic controls (8 male, 12 female, mean age 59.4 years, range 20-90)(Table 5.1). There was no statistical difference between the groups for age or sex.

Skin prick tests, conjunctival biopsy, tissue fixation and processing were performed as previously described.

5.2.2 Immunohistochemistry

Immunohistochemistry was performed as previously described (Chapter 4). The following mouse IgG_1 monoclonal antibodies were used at previously titrated optimal dilutions: AA1 to mast cell tryptase, EG2 to ECP, CD3 to T cells and NP57 to neutrophil elastase (see Table 4.1). Control slides were treated identically, one with omission of the primary antibody, the other with a non-specific IgG_1 monoclonal antibody at the highest concentration of primary antibody used.

TABLE 5.1

Group, age, sex and symptom score of subjects studied for cell marker study

Group	Age	Sex	Score	Group	Age	Sex	Score
Normal	63	F	0	SAC	48	M	7
Normal	90	M	0	SAC	68	F	7
Normal	71	F	0	SAC	23	F	7
Normal	78	M	0	SAC	28	M	8
Normal	51	F	0	SAC	28	M	5
Normal	67	F	0	SAC	72	M	6
Normal	53	F	0	SAC	58	F	6
Normal	82	F	0	SAC	76	M	8
Normal	75	M	0				
Normal	70	M	0	OOS	27	M	0
Normal	74	M	0	OOS	28	F	0
Normal	79	M	0	OOS	35	F	0
Normal	26	F	2	OOS	46	M	0
Normal	21	F	0	OOS	70	M	0
Normal	20	M	0	OOS	24	M	0
Normal	20	F	0	OOS	38	F	0
Normal	20	M	0	OOS	19	M	0
				OOS	47	F	2
				OOS	49	M	0
				OOS	47	F	0
				OOS	74	F	0

Legend

SAC, seasonal allergic conjunctivitis in season; OOS, seasonal allergic conjunctivitis out of season; NOR, normal controls

5.2.3 Quantification and Statistical Analysis

Cells were counted without knowledge of their original source. Only nucleated cells were counted, and all cells within each biopsy were counted at a magnification of x400. The counting protocol described in Chapter 3 was followed.

5.3 Results

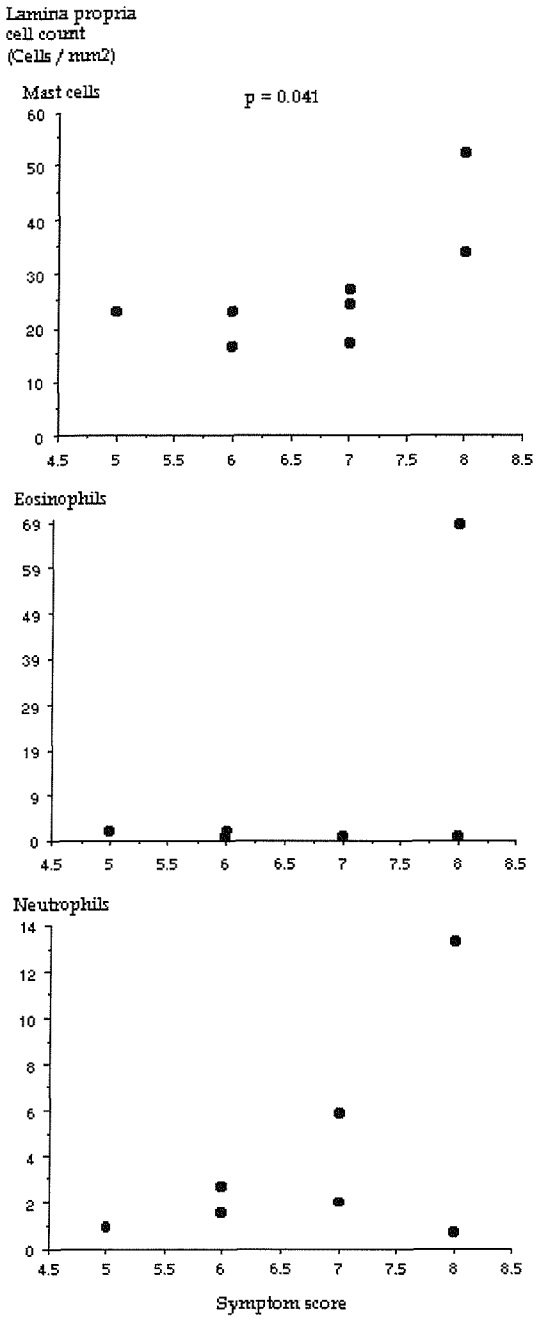
All patients with SAC in season were symptomatic with itch, redness and lacrimation. Patients with SAC in season had a mean symptom score of 6.75 (range 5-8) and all patients with SAC out of season and the normal controls had symptom scores of zero, except one subject in each group whose score was two due to itch. Analysis of symptom scores showed significant differences between SAC in season and normals ($p=0.0001$) and SAC patients in and out of season ($p=0.0002$). No difference in scores existed between normals and SAC patients out of season ($p = 0.94$). The greatest mast cell counts were observed in the patients with maximal symptom scores (Figure 5.1).

High quality histological sections were obtained for cell identification and enumeration (Figure 5.2). Clear immunostaining was present in mast cell, eosinophil and neutrophil cytoplasm with no positive staining obtained in any of the negative control slides (Figure 5.3). Two mast cells were observed in the epithelium of one patient with SAC in season. Mast cells were predominantly located sub-epithelially and around blood vessels. No mast cells or eosinophils were detected in the epithelium of the normal group and in each of two subjects only one neutrophil was observed. No neutrophils were detected in the epithelium of symptomatic patients in season. In only three of these patients were eosinophils observed in the epithelium, a finding which did not reach statistical significance.

The mean mast cell density in normal lamina propria was 17.2 ± SEM 1.2 cells mm^{-2}. Mast cell numbers were elevated significantly in SAC in season (mean 27.62 ± 2.4 cells mm^{-2} ($p=0.042$)). There was no statistical difference between the mast cell count in and out of season in SAC and the mast cell count in SAC patients out of

FIGURE 5.1

The relationship between lamina propria mast cell, eosinophil and neutrophil count and symptom score

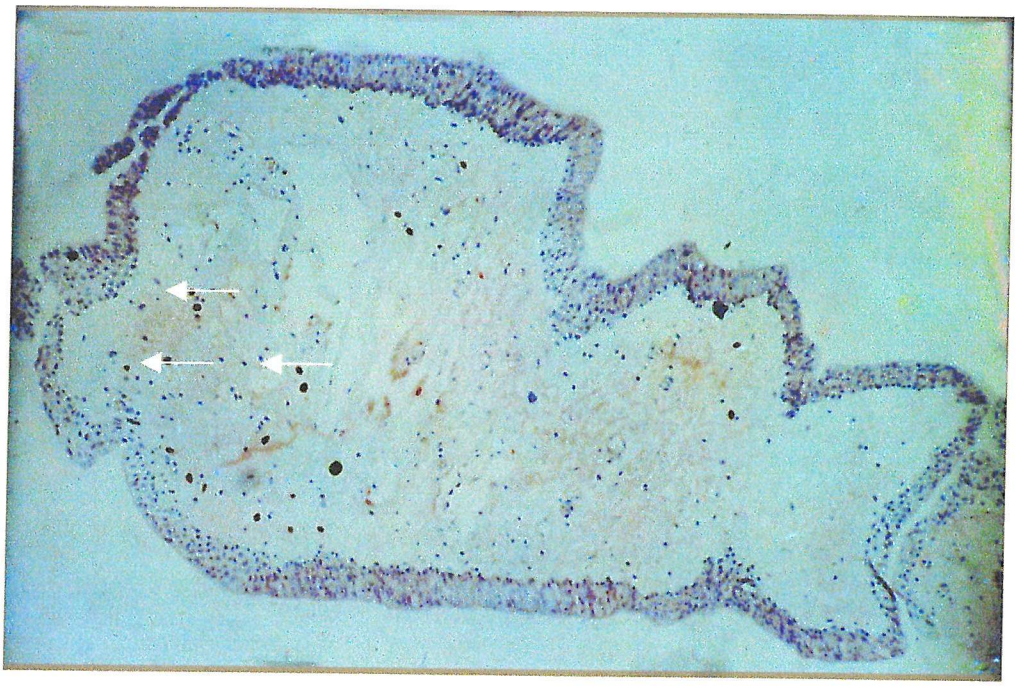


Legend

A positive correlation between symptom score existed for mast cell count only, correlation between neutrophil or eosinophil counts and symptom score was not significant

FIGURE 5.2

Low power photomicrograph of a conjunctival biopsy

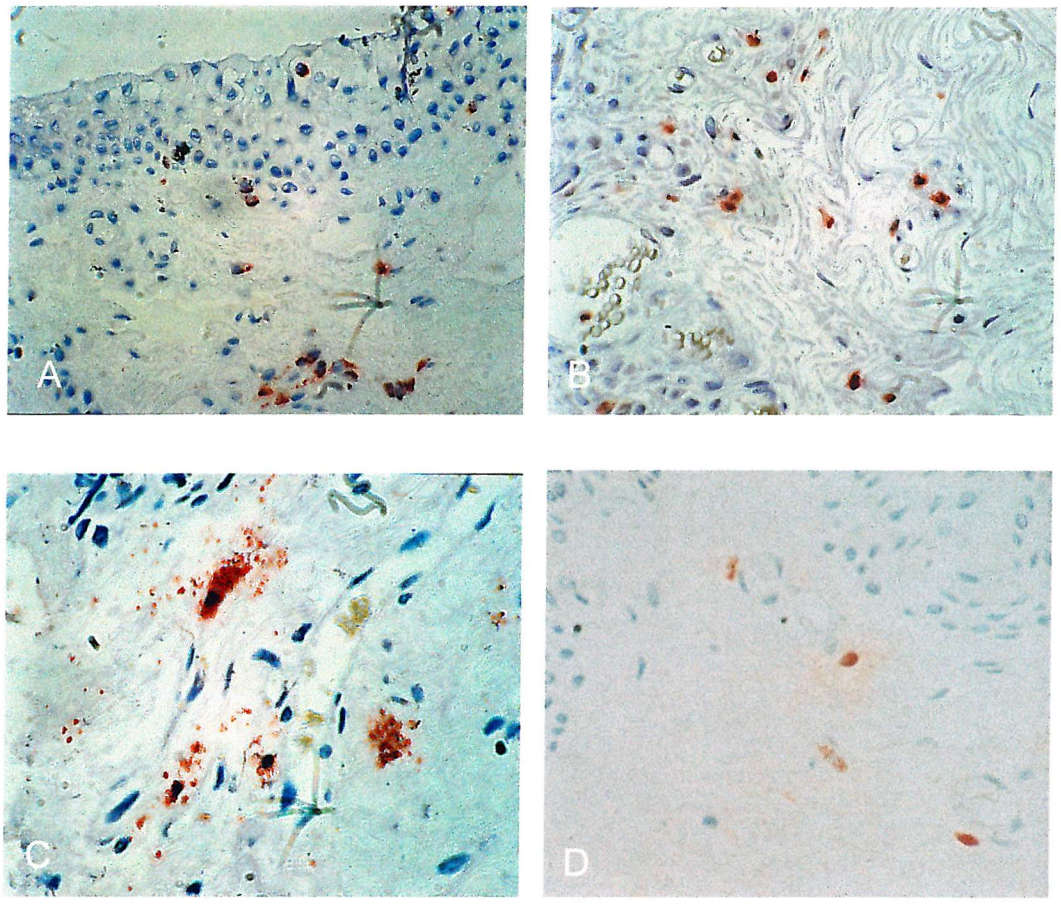


Legend

Epithelium and substantia propria are clearly visible as is positive staining for mAb AA1 (white arrows)(Original mag. X15).

FIGURE 5.3

Immunochemical labeling of mast cells, neutrophils and eosinophils in normal conjunctiva



Legend

Series of 2μm sections of bulbar conjunctiva. Immunohistochemical staining of granulocytes demonstrated by positive staining with the red reaction product AEC.

1A Eosinophils labeled with mAb EG2 (Original mag. x80)

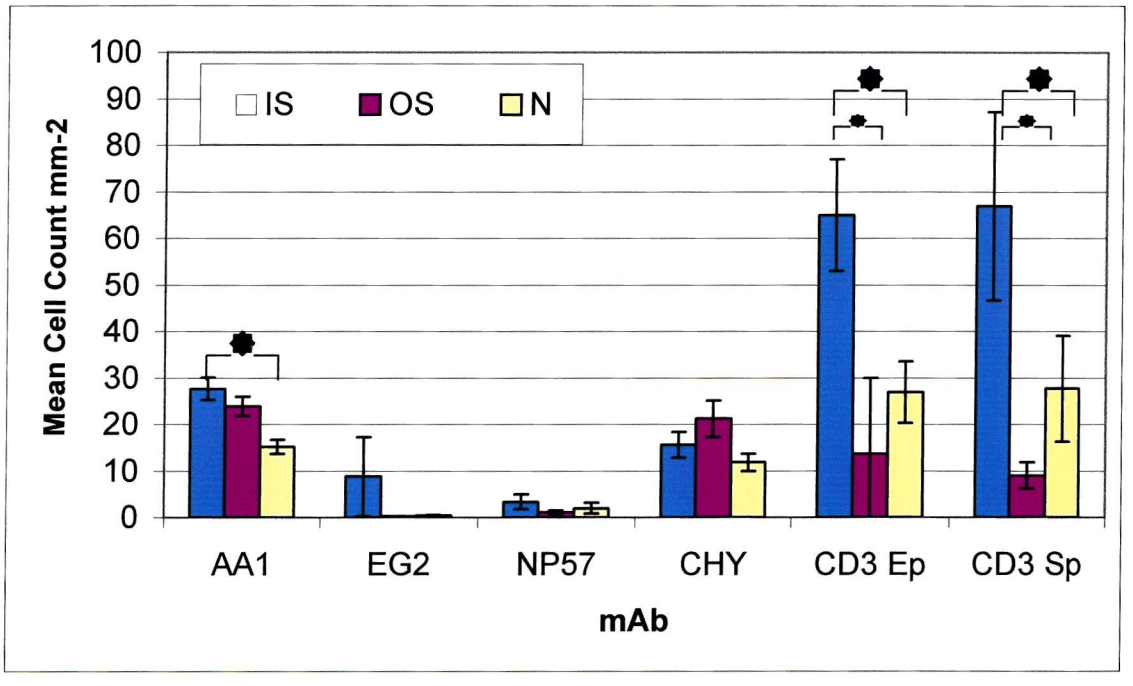
1B Neutrophils labeled with mAb NP57 (Original mag. x102)

1C Mast cells labeled with mAb MAB 1254 to chymase (Original mag. x128)

1D Mast cells labeled with mAb AA1to tryptase (Original mag. x40)

FIGURE 5.4

Mean and SEM counts of tryptase and chymase positive mast cells, neutrophils, eosinophils and T cells in symptomatic and asymptomatic patients with SAC and normal controls



Legend

AA1, tryptase$^+$ mast cells; CHY, chymase$^+$ mast cells; NP57, neutrophils; EG2 eosinophils; CD3 Ep, CD3$^+$ epithelial T cells; CD3 Sp, CD3$^+$ substantia propria T cells. Error bars show SEM's. ⌜✸⌝ $p < 0.05$

season (mean 23.88 ± 2.1 (p=0.243)) was also not significantly different than normals.

Low numbers of neutrophils and eosinophils were found in the conjunctival lamina propria particularly in the SAC subjects, but there was wide inter-subject variation. The mean eosinophil count was 0.39 ± 0.2 cells mm^{-2} in normals and 8.79 ± 8.5 cells mm^{-2} in subjects with SAC in season. There was no significant difference in neutrophil counts in the lamina propria of patients with SAC mean 3.33 ± 1.6 cells mm^{-2} in season compared with both normal (2.79 ± 0.8 cells mm^{-2}) and SAC out of season (1.13 ± 0.4 cells mm^{-2}) (Figure 5.4). Epithelial $CD3^+$ cell numbers were significantly higher in symptomatic patients (64.9 ± 12.0 mm^{-2}) compared with asymptomatic patients (13.6 ± 6.3 mm^{-2}, p=0.014) and normal controls (26.9 ± 6.6 mm^{-2}, p=0.03). $CD3^+$ cell numbers in the substantia propria were also significantly higher in symptomatic patients (66.9 ± 20.3 mm^{-2}) compared with asymptomatic patients (9.0 ± 2.8 mm^{-2}, p=0.0019). There was no statistical difference between $CD3^+$ cell numbers in the substantia propria in asymptomatic (9.0 ± 2.8 mm^{-2}) and normal subjects (27.7 ± 11.4 mm^{-2}).

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

5.4 Discussion

This study demonstrated a significant increase in mast cell numbers in the lamina propria of subjects with SAC during the hay fever season. Although mast cell numbers were higher than normals in subjects with SAC out of season this change did not achieve statistical significance.

Both mast cells and T cells are present in conjunctival tissue but unlike T cells, mast cells are able to store as well as release a T_H2-like panel of cytokines [Plaut 1990] without prior exposure to IL4. Mast cells have been noted to shift phenotype from cells containing the proteases tryptase and chymase (MC_{TC}) to predominantly those containing tryptase only (MC_T) in symptomatic patients with SAC [Baddeley et al. 1995]. This may reflect the selective recruitment of MC_T or the preferential

maturation of these cells in a microenvironment where cells are exposed to repeated allergen exposure, degranulation and subsequent regranulation. Some evidence in rhinitic subjects suggests that mast cell phenotype may regulate the spectrum of cytokines secreted [Bradding et al. 1995a] although the concept of discrete mast cell subsets with particular cytokine secretory patterns has yet to be explored.

No mast cells or eosinophils were found in the conjunctival epithelium of the normal subjects. This is consistent with previous morphological reports [Allansmith et al. 1978] although evidence from conjunctival cytology is conflicting. Some workers have reported low numbers of eosinophils present in scrapings obtained from normal subjects [Kari et al. 1992], but others have failed to demonstrate eosinophils in conjunctival scrapings obtained from patients with VKC even when they were demonstrated histologically in the epithelium [Abelson et al. 1983a]. The lack of increase in eosinophil numbers in the conjunctival lamina propria from subjects with SAC in season is consistent with the mild nature of the inflammation and the absence of sight-threatening complications. Eosinophil activation leads to the release of the arginine rich secondary granule proteins MBP, ECP and eosinophil peroxidase (EPO). MBP and ECP display cytotoxicity for human respiratory epithelial cells [Venge et al. 1988] and MBP has been shown to delay corneal wound healing *in vitro* [Trocme et al. 1994a], and to be deposited in the corneal ulcers associated with VKC [Trocme et al. 1993].

Low numbers of neutrophils have been described previously in the epithelium of normal conjunctival biopsies [Allansmith et al. 1978] although only solitary neutrophils in the epithelium of two subjects were observed. Continued exposure of the conjunctiva to the atmosphere and commensal bacteria whose products are potent chemoattractants for neutrophils [Schiffmann et al. 1975], may explain the presence of neutrophils in an otherwise healthy tissue. In patients with SAC studied in season, no significant increase in neutrophil numbers was found in the lamina propria when compared to SAC out of season or normal subjects. Overall this evidence suggests that neutrophil infiltration does not play a significant role in SAC during the seasonal period of allergen exposure.

In summary, this work provides the first account of the tissue changes in bulbar conjunctival mast cell, eosinophil, neutrophil and T cell numbers in SAC using immunohistochemistry. A significant rise in mast cell numbers in the lamina propria but not epithelium of symptomatic subjects with SAC was observed, but no significant increase in either neutrophil or eosinophil numbers either in the substantia propria or epithelium. T cell numbers were significantly higher in both epithelium and substantia propria in symptomatic SAC subjects. These observations suggest that there are fundamental differences in the pathogenesis of SAC, in which sight threatening complications do not occur, and the chronic forms of allergic conjunctivitis in which a principally eosinophilic inflammation is associated with the potential for corneal blindness.

CHAPTER 6

Conjunctival mast cell cytokines

6.1 Introduction

Mast cells are recognized as the central effector cells in SAC through their capacity to rapidly release histamine and other stored and newly synthesized mediators. Chapter 5 reported increased conjunctival mast cell numbers during symptomatic disease and elevated levels of the mast cell products, histamine, tryptase and LTC_4 are detectable in tears [Abelson et al. 1980; Bisgaard et al. 1985; Butrus et al. 1990]. Mast cell activation in SAC leads to a well described early phase response (EPR) corresponding to the Type I hypersensitivity reaction triggered by aggregation of the multivalent FcεRI receptor by allergen. Allergen challenge studies have, however demonstrated a late phase response (LPR) characterized by the accumulation of eosinophils and neutrophils [Abelson et al. 1980; Bonini et al. 1987; Bonini et al.1988; Butrus et al.1990] which is inconsistent although reproducible using high dose allergen challenge [Anderson 1996]. Topical application of histamine [Ciprandi et al.1993a], PGD_2 [Abelson et al.1985], LTD_4 and LTC_4 reproduces signs of allergic conjunctivitis in animals and humans [Woodward et al.1985], these mediators are also chemotactic for eosinophils and neutrophils [Spada et al.1986; Woodward et al.1986]. T_H2-like cytokines however, are known to play key roles in upregulating the allergic response in susceptible individuals and although T helper cells have traditionally been considered to be the principal source of these molecules, evidence for the role of the mast cell in cytokine production is emerging. Cytokines are immunoregulatory molecules produced by, and acting on, all cells of the immune system. Their function is to mediate cell to cell signaling so that tissue effector responses are co-coordinated. Cytokines are known by a variety of names which may reflect their source (e.g. lymphokines and monokines were named according to their production by lymphocytes and monocytes), properties (e.g. chemokines were so-named because of their chemotactic properties), or order of discovery. Other names for these molecules include the interleukins (IL's) and haematopoietic growth factors. Cytokines are pleiotropic and

because their functions were noted to be modulated by the presence of other cytokines, the term 'cytokine network' [Balkwill et al. 1989] arose to describe their actions *in vivo*. Cytokine receptors fall into several major classes: the immunoglobulin superfamily, haematopoietin-receptor family, tumour necrosis factor (TNF) family and chemokine-receptor family. The genes coding both receptors and cytokines are closely linked, sharing both structural and functional similarities. IL-3, IL-4, IL-5, IL-13 and GM-CSF are all coded for by genes on the short arm of chromosome 5, and all are secreted by T_H2 cells. Genetic studies in Amish families indicate that five markers on chromosome 5q31.1 including the IL-4 gene (*IL4*) and a gene controlling total serum IgE concentration are linked [Marsh et al. 1994] suggesting that IgE production may be regulated by *IL4* or a nearby gene in this region. Components of cytokine receptors may also be closely related or identical allowing a degree of functional overlap. IL-4 and IL-13 share nearly identical functions except that IL-13 has no known biological effect on T cells. The IL-4 receptor (IL-4-R) found on T cells, B cells, mast cells, basophils and others, comprises an integral membrane glycoprotein with a large intracellular domain. Signal transduction takes place through the IL-4R, an 825 amino acid membrane glycoprotein of which 560 amino acids are intracellular. Multiple regions of the intracellular chain are used to induce proliferative and gene expression activity through the binding of Janus family tyrosine kinanses (JAK-1 and JAK-3) to membrane proximal regions and the tyrosine phophorylation of insulin receptor substrates (IRS)-1 and IRS-2 through the I4R motif distal to this region [Ryan 1997]. Activation of the signal transducer and activator of transcription (STAT)-6 is mediated by the gene expression domain, a more distal region of the receptor, an element key in inducing germline Cε activation and the isotype switching of B cells toward IgE production. The IL-4Rα chain is common to both the IL-4R and IL-13R, and although IL-4 is considered the critical cytokine in inducing IgE class switching, IL-13R ligation may also play an important role [Emson et al. 1998].

The cytokine environment in which T cells develop has been shown to influence the nature of T_H cell produced. Murine experiments demonstrated that IFN-γ stimulated the preferential development of T_H1 cells whilst IL-4 stimulated T_H2 cell development. IL-12 and IFN-γ produced by macrophages in humans has been demonstrated to have similar effects, whereas low IFN-γ concentrations and IL-4 promote the development of the T_H2 phenotype [Romagnani 1992]. Other cytokines elicit responses important in immune

responses and particularly in allergic immune responses. These are usually achieved through local actions, but some e.g. IL-3, IL-5 and GM-CSF act in bone marrow to upregulate myelopoiesis to stimulate the production, growth and differentiation of macrophages and granulocytes.

The stimulation of autocrine cytokine production is a key component of both T cell activation and proliferation and B cell differentiation. IL-2 stimulates T cells to enter the cell cycle and its synthesis is stimulated by CD28/B7 interaction. IL-6 is required for B cell activation and class switching to IgE production, activation of the nuclear transcription factor for IL-6 gene expression, NF-κB, has been demonstrated to follow IL-4 and CD40 signaling of purified B cells [Jeppson et al. 1998].

Attention has traditionally focused on T lymphocytes as the major source of cytokines. Evidence for mast cells as a potential source for cytokines was first provided by the Abelson murine leukaemia virus (A-MuLV)-transformed tumourigenic mouse mast cell line which constitutively expressed granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA and released GM-SCF protein [Chung et al.1986]. Later, Gordon *et al.*[Gordon et al.1990a] demonstrated that non-transformed or freshly isolated mouse mast cells in culture constitutively contained TNFα and produced high levels of TNFα on IgE-dependant stimulation. Non-transformed mouse mast cell lines were subsequently shown to secrete a panel of cytokines (IL-3, IL-4, IL-5 and IL-6) in response to cross-linkage of FcεR1 [Plaut et al.1989]. Evidence that human mast cells were a source of cytokines was demonstrated in mast cells from the respiratory tract which contain both IL-4 and rapidly release IL-4 bioactivity when stimulated with anti-IgE [Bradding et al.1992]. Although T cells have been demonstrated to contain mRNA for a number of cytokines, T cell cytokine product has not been demonstrated at multiple tissue locations [Barata et al.1998; Bradding et al.1992]. Lacking an abundant cytoplasm, T cells may not contain stores of cytokine product, but synthesize these molecules for rapid export. The source of the priming pulse of IL-4 required for T_H2 cell development and subsequent activation to secrete the panel of typical chromosome 5q31 cytokines remains to be determined.

Evidence for mast cell production and release of cytokines was first obtained in rodent studies followed by transformed human mast cell lines. More recently IL-4 and IL-8

production by non-transformed human mast cells from the respiratory tract [Bradding et al.1992; Moller et al.1993] and the demonstration of immunoreactivity for IL-5, IL-6 and IL-8 in nasal and bronchial mucosa [Bradding et al.1993; Bradding et al. 1994] strongly suggested a role for this cell in cytokine production *in vivo* and therefore the capacity to participate in local tissue immunoregulation. IL-4 is a critical regulator of IgE production [Marsh, Neely, et al. 1994] and a potent T cell growth factor [Spits et al. 1987] although T cells themselves require a priming pulse of IL-4 for the generation of IL-4 production [Le Gros et al. 1990]. IL-5 is a selective regulator of eosinophil function [Yamaguchi et al. 1991] and IL-6 is recognized to stimulate T cell and mast cell growth and to induce plasma cell differentiation of human B cells [Hirano et al. 1986]. The chemokine IL-8 and cytokine TNFα are important in selective neutrophil and eosinophil recruitment through chemotactic and adhesion molecule upregulation [Bevilacqua 1993; Shute 1994; Walsh et al.1991]. The detection of cytokines in tears has been shown to be elevated in allergic conjunctivitis [Fujishima et al.1995; Uchio et al.2000] but the source of the cytokine is unknown and the biological significance of high levels of a locally acting mediator in tears unknown. For these reasons and because of the importance of these cytokines in the upregulation of immune responses the present studies were undertaken to seek evidence of immunoreactivity for key T_H2-like cytokines in human conjunctival tissue in subjects with symptomatic and asymptomatic disease as well as normal controls. In-situ hybridization for cytokine message was also investigated.

6.2 Methods

6.2.1 Subjects

Conjunctival biopsies were obtained from 8 atopic patients with SAC in season, 8 atopic patients with SAC out of season (see Table 6.1 for age, gender distribution and symptom scores) and 14 non-atopic controls (mean age 67.1 range 26-91 years).

Subjects for the double IHC and dual ISH/IHC studies are described in Table 6.2. The mean symptom score for patients in season was 6.75 (range 5-8), this differed significantly from the symptom scores in both the normal and out of season group in whom one patient in each group had a score of two due to itch, but all other subjects scored zero ($p<0.0001$).

TABLE 6.1

Group, age, sex and symptom score of subjects studied for cytokine counts

Group	Biopsy code	Age	Sex	Score	Group	Biopsy code	Age	Sex	Score
Normal	63	63	F	0	OOS	45	27	M	0
Normal	64	90	M	0	OOS	53	46	M	0
Normal	67	71	F	0	OOS	65	70	M	0
Normal	68	78	M	0	OOS	66	24	M	0
Normal	100	51	F	0	OOS	69	38	F	0
Normal	101	91	F	0	OOS	70	19	M	0
Normal	103	67	F	0	OOS	93	47	F	2
Normal	106	53	F	0	OOS	95	49	M	0
Normal	110	82	F	0	SAC	78	48	M	7
Normal	112	75	M	0	SAC	79	68	F	7
Normal	113	70	M	0	SAC	80	23	F	7
Normal	114	74	M	0	SAC	81	28	M	8
Normal	121	26	F	2	SAC	82	28	M	5
Normal	122	48	F	0	SAC	83	72	M	6
					SAC	84	58	F	6
					SAC	85	76	M	8

Legend

SAC, seasonal allergic conjunctivitis in season; OOS, seasonal allergic conjunctivitis out of season; NOR, normal controls

TABLE 6.2

Group, age, sex and symptom score of subjects studied for cell cytokine co-localization study

Group	Biopsy code	Age	Sex	Score	Group	Biopsy code	Age	Sex	Score
Normal	63	63	F	0	OOS	45	27	M	0
Normal	64	90	M	0	OOS	53	46	M	0
Normal	67	71	F	0	OOS	65	70	M	0
Normal	68	78	M	0	OOS	66	24	M	0
Normal	101	91	F	0	OOS	69	38	F	0
Normal	103	67	F	0	OOS	70	19	M	0
Normal	114	74	M	0	OOS	93	47	F	2
Normal	121	26	F	2	OOS	95	49	M	0
Normal	122	48	F	0					
SAC	78	48	M	7	SAC	82	28	M	5
SAC	79	68	F	7	SAC	83	72	M	6
SAC	80	23	F	7	SAC	84	58	F	6
SAC	81	28	M	8	SAC	85	76	M	8

Legend

SAC, seasonal allergic conjunctivitis in season; OOS, seasonal allergic conjunctivitis out of season; NOR, normal controls

6.2.2 Immunohistochemistry

This was performed on 2μm sections of GMA embedded tissue using the protocol previously described (Chapter 4) using the a panel of monoclonal antibodies to IL-4, IL-5, IL-6, IL-8, SCF and TNFα, the details of which are given in Table 4.1. Negative controls comprised omission of the primary antibody and an unrelated IgG1 mAb.

6.2.3 Double immunohistochemistry

4μm sections were cut and treated as above. Primary antibodies to mast cell tryptase (AA1) and IL-4 (3H4) were applied as before and labeled with either SBP with fast red as a chromogen, or fluoroscein isothiocyanate (FITC) anti-mouse (Dako Ltd, UK.) mAb. Propidium iodide was used as a counterstain and the cells detected using a confocal laser microscope (Leica, UK). Stringent controls were performed to ensure the specificity of the detection, with the omission of each primary antibody and separately the omission of each secondary antibody followed by application of both detection systems.

6.2.4 *In-situ* hybridization

Synthesis and labeling of the IL-4 riboprobe was performed as previously described (Chapter 4). The protocol for ISH (Section 4.4) was followed. Negative control slides comprised using the sense IL-4 riboprobe and omission of riboprobe. Positive controls were performed using a 300 base pair riboprobe for the constitutive cytoskeletal protein β-actin (Ambion, Oxford, UK).

6.2.5 Double ISH-IHC

The ISH protocol was followed by immunohistochemistry employing the mAb AA1 to mast cell tryptase as above. Control slides were treated identically and comprised a combination of sense probe and irrelevant antibody controls performed as part of the individual protocols for IHC and ISH above.

6.2.6 Quantification and statistical analysis

Cytokine positive cells were counted and data described and analyzed using the protocols described in Section 4.2.

6.3 Results

One patient developed an inflammatory mass involving Tenons' capsule and conjunctiva following conjunctival biopsy. This resolved completely on topical medication (Figure 6.1).

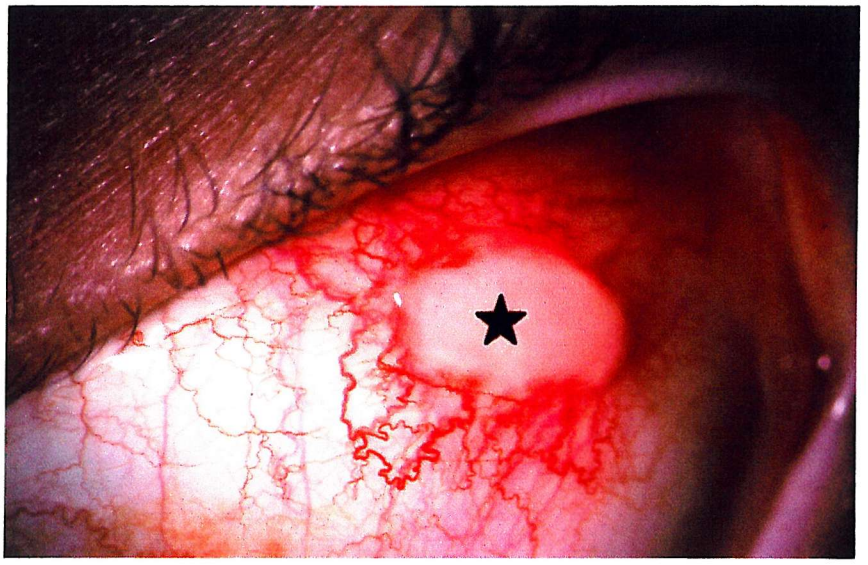
The mAb 3H4 against IL-4 gave a characteristic ring staining pattern in most cells although some cytoplasmic staining was noted. The mAb 4D9 gave a predominantly cytoplasmic staining pattern although some cells were stained around their periphery. In sequential sections both 3H4 and 4D9 stained the same cell (Figure 4.2). The mAb's MAB7 to IL-5, 104B11 to IL-6 and 52B83 to TNFα, gave clear cytoplasmic staining whilst 2D7 against IL-8 gave faint cytoplasmic staining but marked epithelial staining (Figure 6.2). Some cells displayed weak staining for cytokine with only a few granules stained for product, the majority however, were strongly positive. Examination of sequential sections enabled the identification of the same cells to be performed using morphological landmarks and the co-localization of cytokine product to mast cell tryptase (Figures 6.3 and 6.4). In some cases, immunoreactivity of positive granules could not be co-localized to nucleated cells and these were not counted.

6.3.1 Cytokine counts

The total, mean ± SEM cytokine counts are shown in Figure 6.5. No positive cells were noted in any group for IL-8 although strong epithelial staining was present in all groups. Using the Kruskell Wallis test there were significant statistical differences across the groups for IL-4 using the mAb 3H4 ($p = 0.0002$) and IL-6 ($p=0.04$) with no statistical difference between the groups for IL-4 using the mAb 4D9, IL-5, IL-6, SCF or TNFα. The number of 3H4 positive cells was significantly elevated in symptomatic patients (19.3 ± 2.9 mm^{-2}) vs. normals (6.2 ± 1.5 mm^{-2})($p = 0.0013$) but not compared with

FIGURE 6.1

Complication of conjunctival biopsy

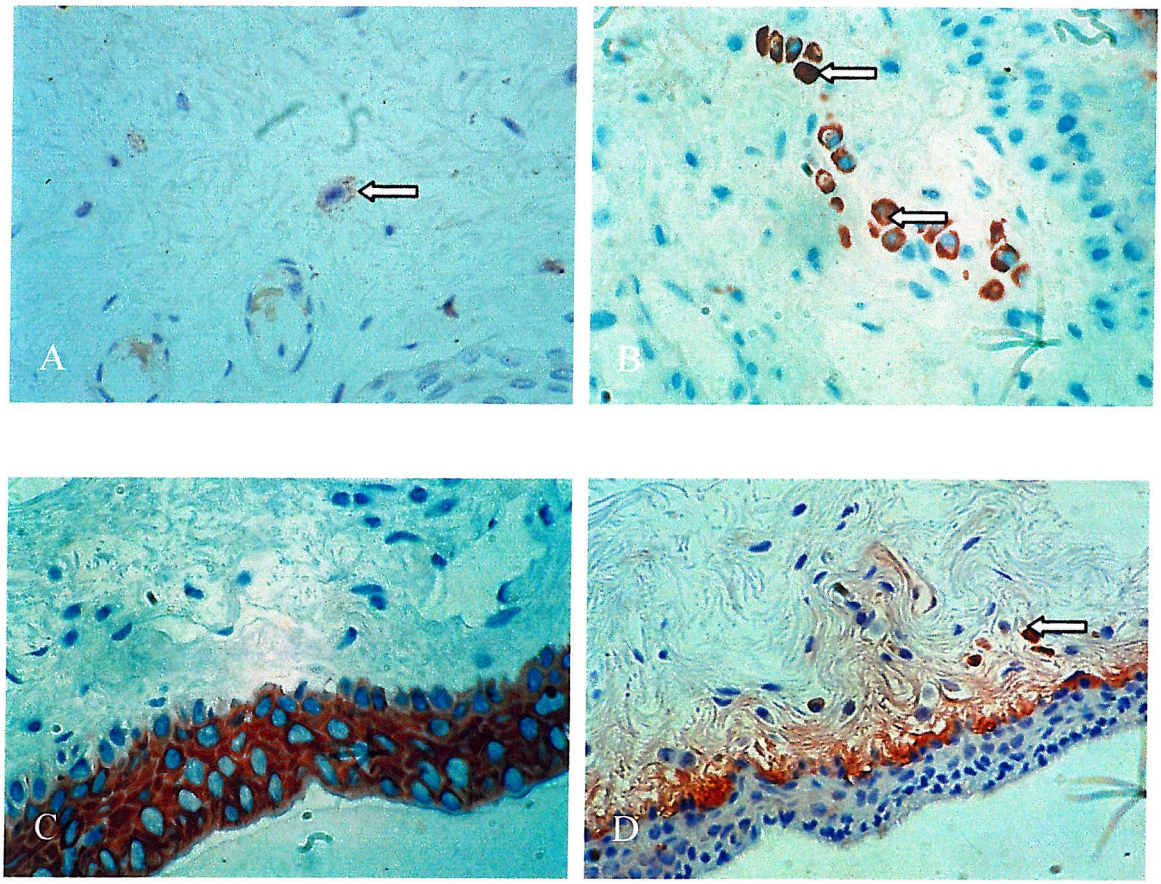


Legend

Inflammation of Tenons capsule (black star ★) occurred following one bulbar conjunctival biopsy. The inflammation resolved completely after 10 days and topical antibiotic treatment.

FIGURE 6.2

Composite images to show immunostaining of mast cell cytokines in human conjunctival biopsies.

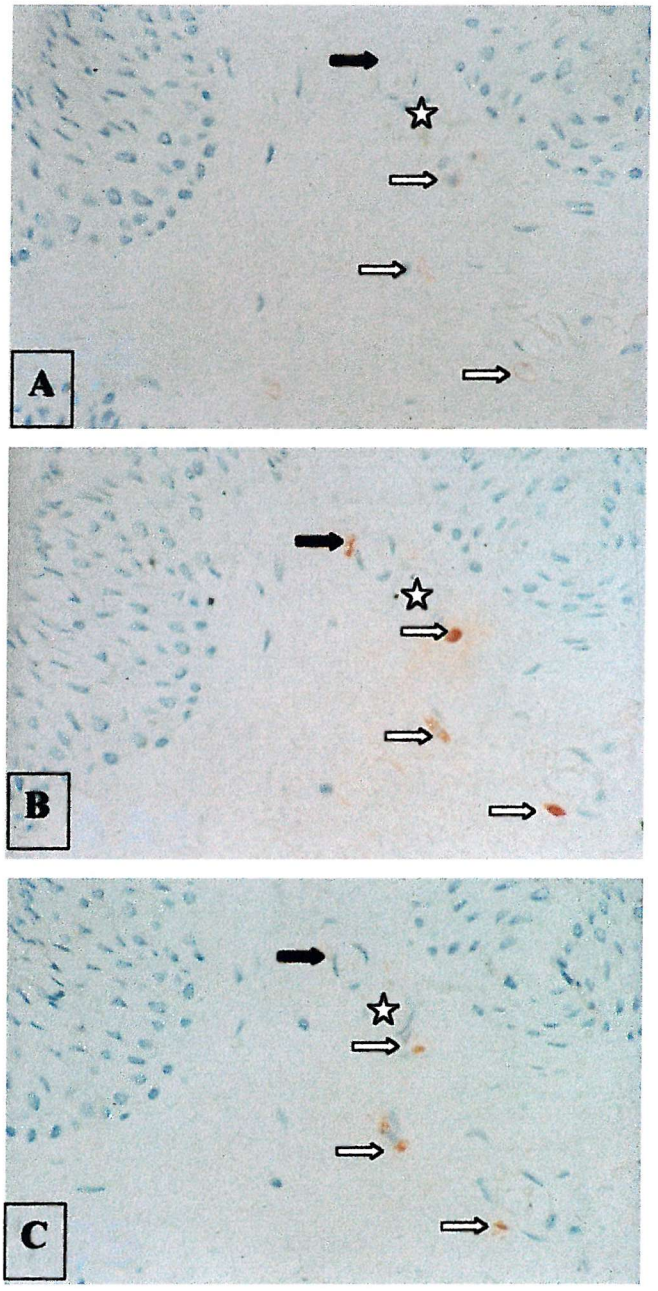


Legend

Immunohistochemistry on 2μm conjunctival sections. Positive staining for: A) IL-13 using the mAb MAB213 (white arrows (Original mag. x40), B) IL-6 using mAb 104B11 (Original mag. x128), C) IL-8 using mAb 2D7 (Original mag. x 128) showing dense epithelial staining, and D) TNFα using the mAb 52B83 (Original mag. x40).

FIGURE 6.3

Sequential immunohistochemical staining for mast cell tryptase and IL-4

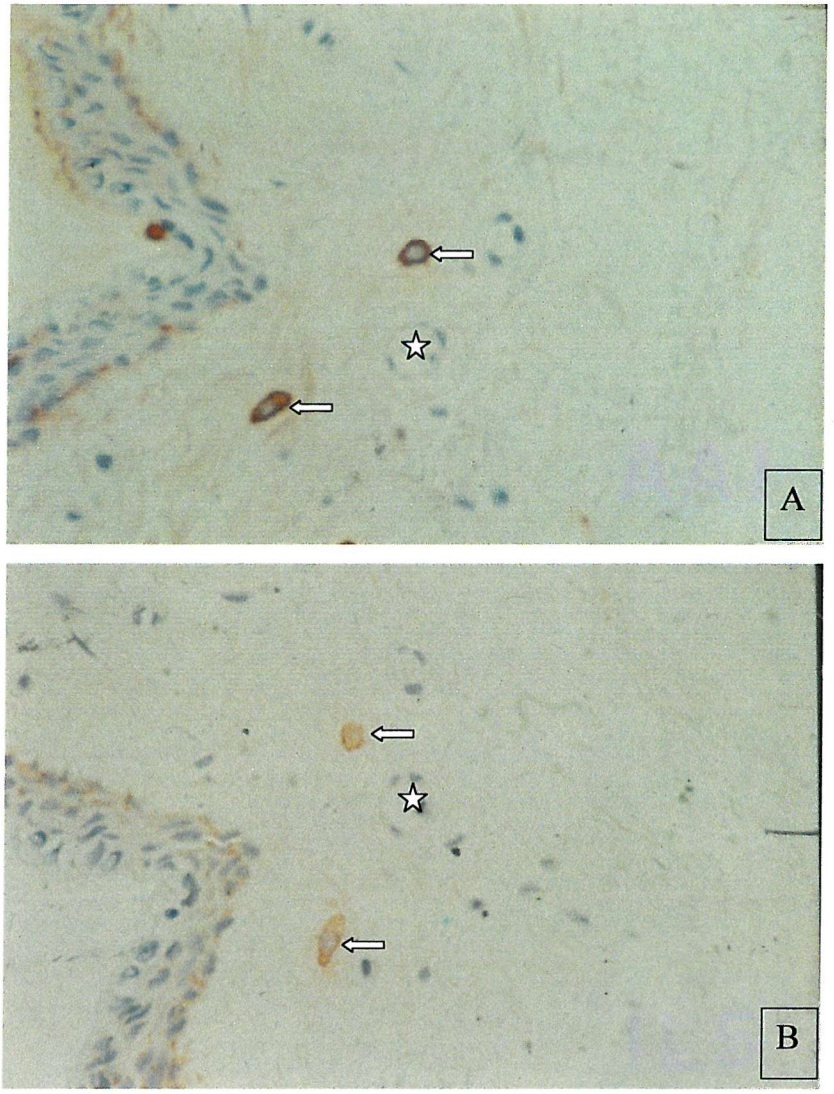


Legend

Immunochemistry on sequential sections 2μm apart. **6.2 a** ☆show corresponding vessels in each section used in addition to epithelial contour to align sequential acetate sheets. Positive staining for IL-4 using the mAb 3H4 (white arrows) is seen in A) to correspond to positive staining for mast cell tryptase (white arrows) B) and C) IL-4 using the mAb 4D9 (white arrows). Mast cells in B) (black arrows) did not co-localize with IL-4 immunoreactivity (black arrows) in corresponding sections. **6.2 b** Sequential immunoreactivity for mast cell tryptase using the mAb AA1 A) and IL-5 B) in adjacent section. (Original mag. x40)

FIGURE 6.4

Sequential immunohistochemical staining for mast cell tryptase and IL-5



Legend

Immunochemistry on sequential sections 2μm apart. ☆ show corresponding vessels in each section used in addition to epithelial contour to align sequential acetate sheets. Positive staining for tryptase using the mAb AA1 (white arrows) is seen in A) to correspond to positive staining for IL-5 using the mAb MAB7 (white arrows) B) (Original mag. x128)

asymptomatic patients (17.4 ± 2.2 mm^{-2}). The difference between asymptomatic and normal controls was also significant (p=0.0004). The number of IL-6 positive cells was also significantly elevated in symptomatic patients (8.0 ± 5.1 mm^{-2}) vs. normals (2.0 ± 1.0 mm^{-2})(p = 0.015) and also compared with asymptomatic patients (2.5 ± 1.4 mm^{-2}). The difference between asymptomatic and normal controls was not significant. Although there were differences in the numbers of cytokine positive cells for IL-5, TNFα, SCF and IL-13 these changes did not achieve statistical significance.

6.3.2 Co-localization results

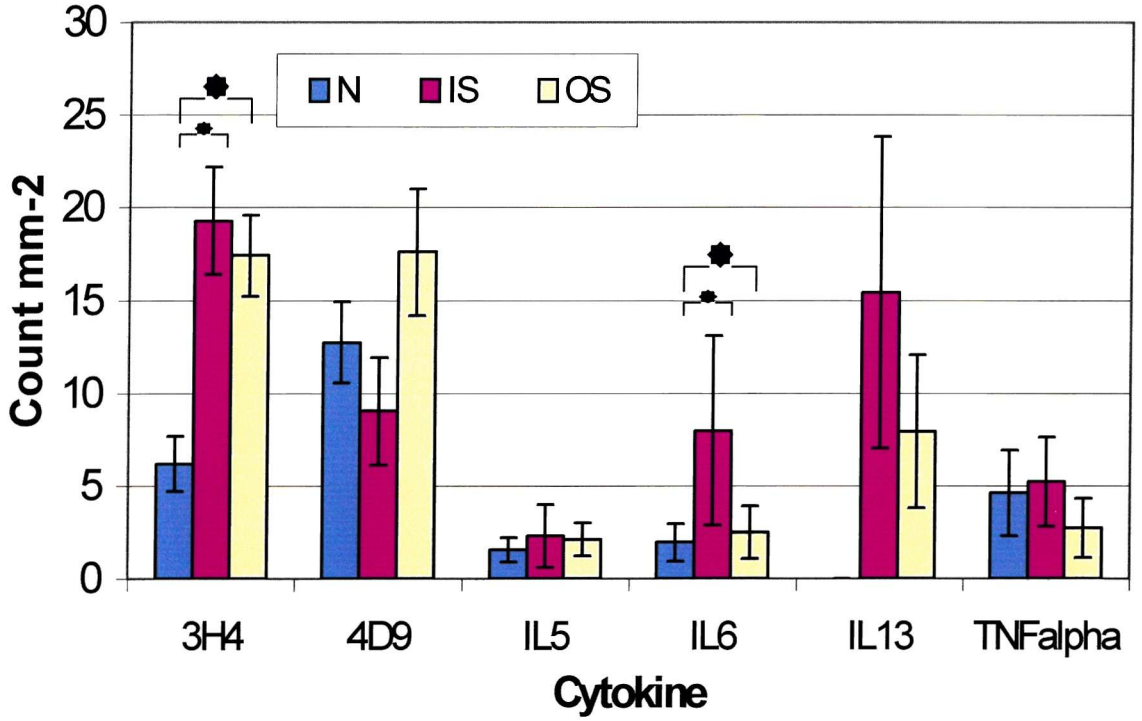
The proportion of cytokine positive cells which were mast cells is given in Figure 6.6. The mAb 3H4 to IL-4 and AA1 co-localized in 19 (76%) of all subjects and to a significantly higher proportion of mast cells in subjects with active SAC than asymptomatic subjects (p=0.016). mAb 4D9 to IL-4 and AA1 co-localized in about the same proportion of subjects (80%) but there was no significant difference between the groups. IL-5, IL-6, TNFα and IL-8 co-localized to mast cells in substantially fewer patients. IL-5 was localized to one patient from each group, IL-6 to six patients from all groups and IL-8 from two patients only, one symptomatic and the other asymptomatic from SAC. No statistically significant difference between the groups with respect to the proportion of mast cells containing each of the above cytokines. Immunofluorescence studies using the confocal microscope demonstrated cytoplasmic distribution of mast cell tryptase at high power (Figure 6.7) with a clear ring staining pattern for IL-4 using the mAb 3H4 (Figure 6.8). Double IHC demonstrated the simultaneous presence of IL-4 using the mAb 3H4, and tryptase within the same cells in biopsies taken from subjects with symptomatic SAC and normal controls (Figure 6.9).

6.3.3 In-situ results

In-situ hybridization demonstrated clear labeling of IL-4 mRNA within conjunctival sections with no staining in the sense probe controls (Figure 6.10). Double ISH - IHC clearly labeled AA1 positive cells which were also IL-4mRNA positive (Figure 6.11). Using this method 75.8% of mast cells were positive for IL-4 mRNA in normals with

FIGURE 6.5

Mean cytokine counts for subjects with symptomatic and asymptomatic SAC and normal controls



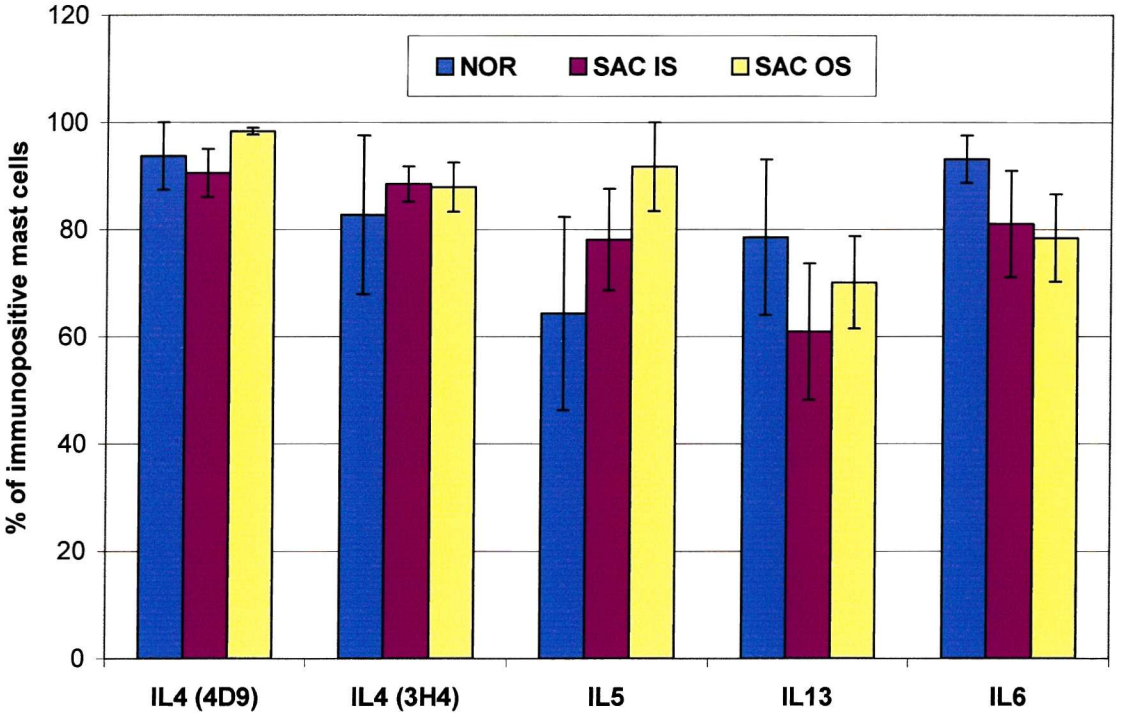
Legend

NOR, normal controls; SAC IS, symptomatic SAC subjects in season; SAC OS, asymptomatic SAC subjects out of season. Error bars show SEM for each count.

$P < 0.05$

FIGURE 6.6

Percentages of cells immunopositive for a panel of cytokines which were also identified as mast cells in symptomatic and asymptomatic patients with SAC and normal controls

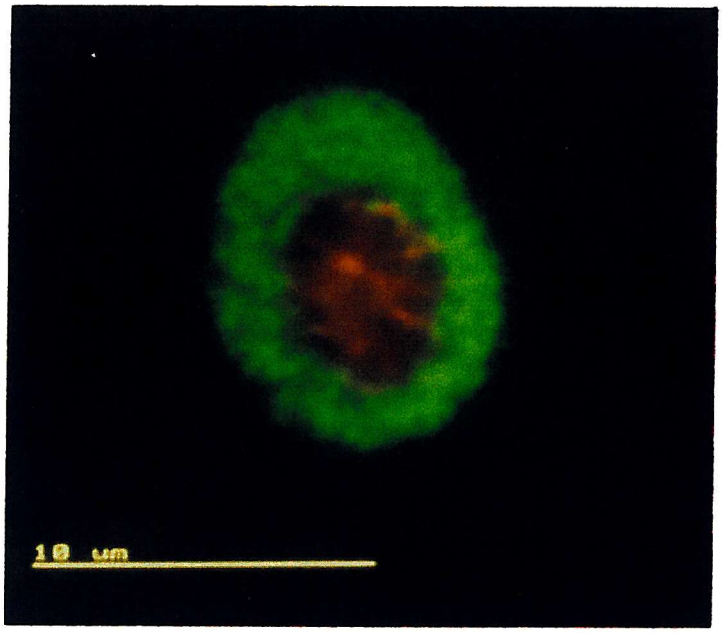


Legend

NOR, normal controls; SAC IS, symptomatic SAC subjects in season; SAC OS, asymptomatic SAC subjects out of season. Error bars show SEM for each count.

FIGURE 6.7

Confocal photomicrograph showing immunofluorescent labeling of mast cell tryptase

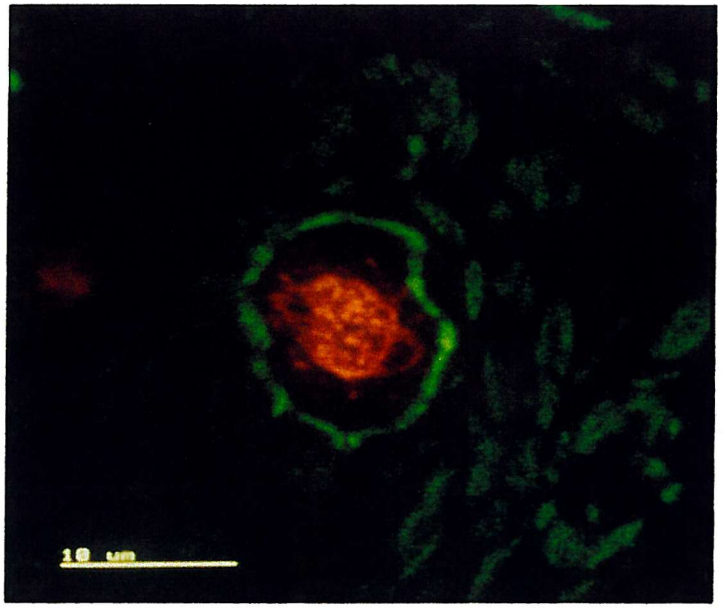


Legend

The mAb fast red labeled mAb AA1 is detected as bright green cytoplasmic fluorescence with propidium iodide nuclear counterstaining (Magnification x 640). Scale bar depicts 10μm.

FIGURE 6.8

Confocal photomicrograph showing immunofluorescent labeling of IL-4

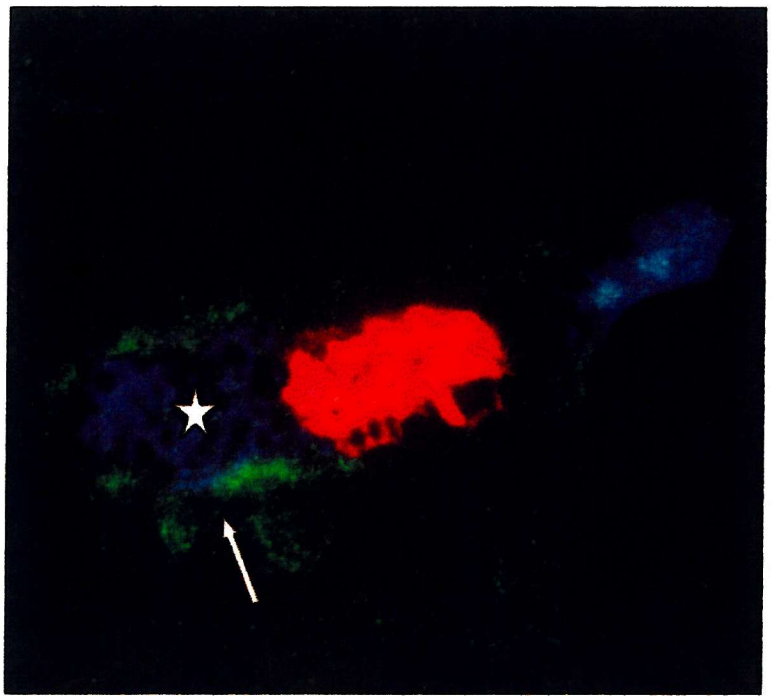


Legend

The FITC labeled mAb 3H4 for IL-4 is detected as a bright green fluorescent ring around the nucleus counterstained with propidium iodide (Magnification x 640). Note high levels of non-specific background fluorescence. Scale bar depicts 10μm.

FIGURE 6.9

Confocal photomicrograph to show double immunofluorescent labeling for IL-4 and tryptase

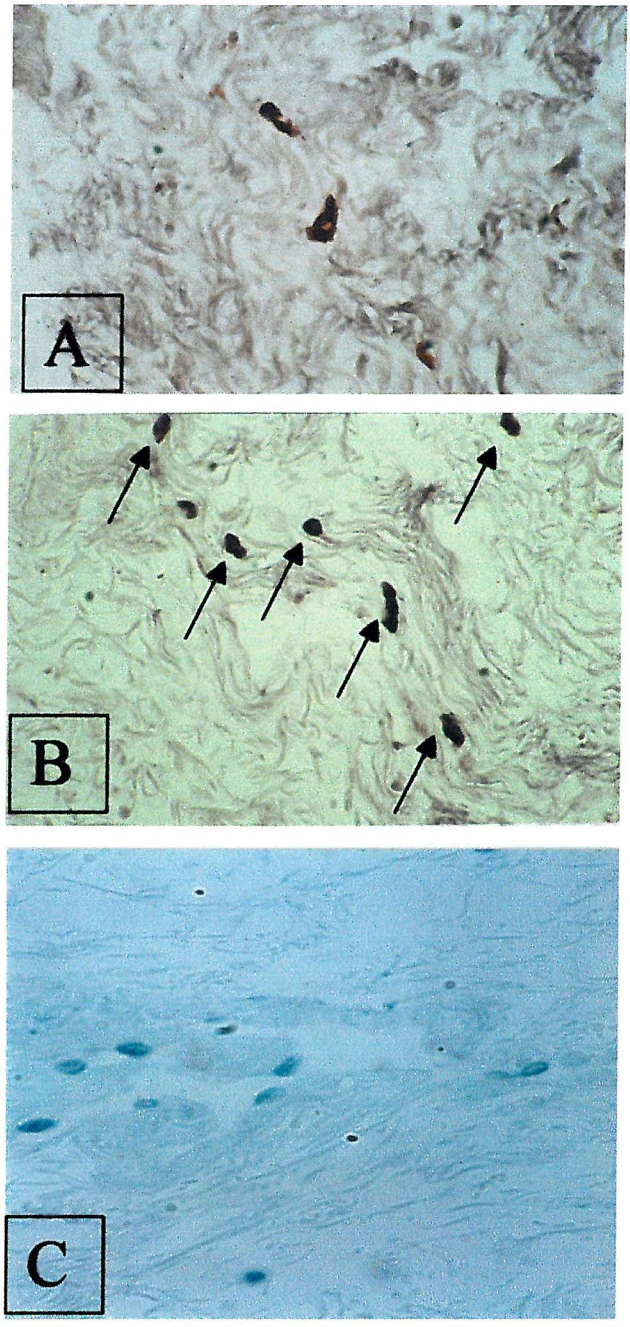


Legend

Simultaneous visualization of mast cell tryptase (white star) using mAb AA1 and IL-4 (white arrow) using the mAb 3H4 (Magnification x 640).

FIGURE 6.10

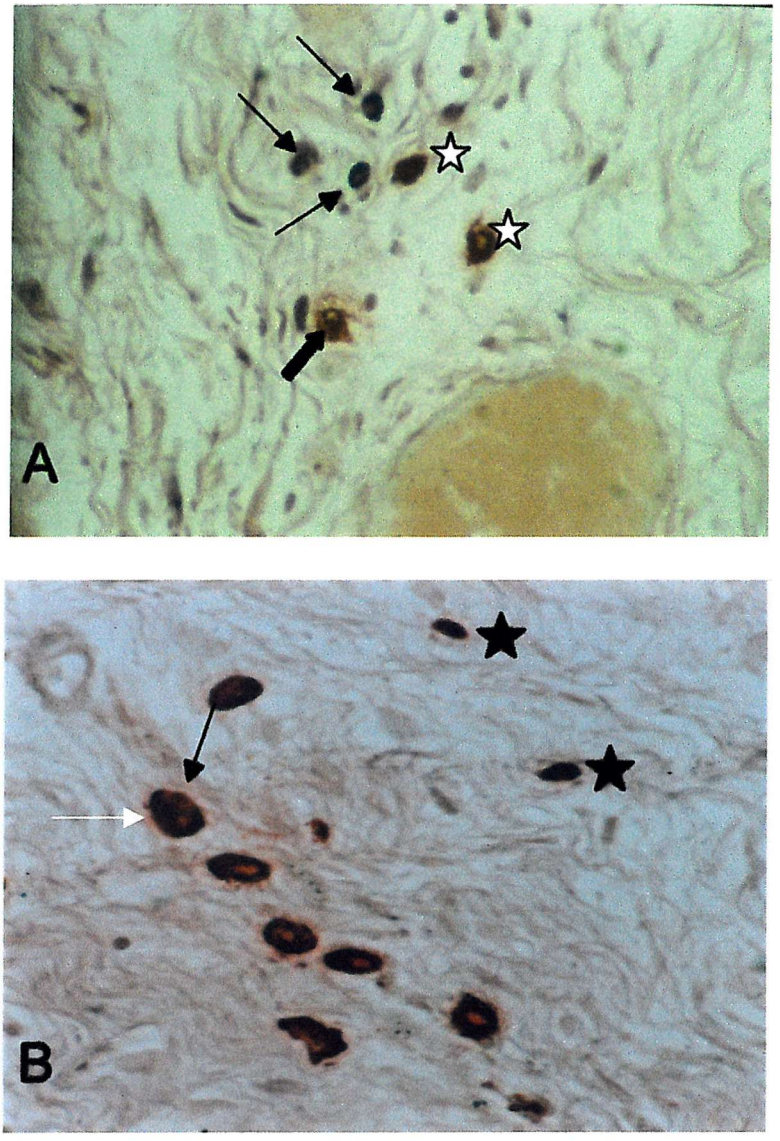
Photomicrograph of IL-4 mRNA$^+$ cells and control staining



Legend
Initial *in-situ* hybridization experiments resulted in high levels of tissue digestion A) with poor specificity of positive staining, later experiments allowed clear identification of B) dark blue staining of IL-4 mRNA$^+$ cells using IL-4 antisense riboprobe (arrows) compared with C) sense (negative) control. (Original mag. x40)

FIGURE 6.11

Photomicrograph of dual ISH and IHC of mast cell tryptase and IL-4



Legend

Dark thin arrows show IL-4 mRNA$^+$ cells stained with the blue chromogen NBT-BCIP, thick black arrow shows an AA1$^+$ mast cell stained with the red chromogen AEC, white stars show simultaneous IL-4 mRNA$^+$ AA1$^+$ cell staining (Original mag. x 128)

78.7% positive in symptomatic SAC subjects. 62% of IL-4 mRNA$^+$ cells in normals were tryptase$^+$, this fell to 42.5% in symptomatic SAC subjects (p=ns).

6.4 Discussion

This study showed conjunctival mast cells to be a source of IL-4, IL-5, IL-6, IL-8, IL-13 and TNFα. Mast cells were noted to be the predominant source of IL-4 and IL-6 in normal subjects whereas few mast cells appeared to store IL-5, IL-8 or TNFα. The significant rise in the proportion of mast cells containing IL-4 demonstrated by the mAb 3H4 during symptomatic SAC (p=0.0157) suggests that this cell may be an important source of this cytokine during allergic inflammation. The difference in the numbers of cells and the pattern of staining obtained with the different mAb's to IL-4 was noteworthy. The mAb 3H4 gave a predominantly ring staining pattern although the exact location of this staining to intracellular, membrane or extracellular domain could not been determined. The mAb 4D9 gave a predominantly cytoplasmic staining pattern although in some cells, ring staining was observed. A small proportion of cells were observed to stain with both mAb's. The difference in pattern between ring and cytoplasmic staining may have reflected a conformational change in the IL-4 molecule leading to changes in the availability of IL-4 epitopes. This change might be caused by the export of IL-4 from the cell and has been well described for other cytokines e.g. TGFβ-1 in which a similar pattern of staining is observed [Flanders et al. 1989] and would be consistent with the observed increase in 3H4$^+$ cells in symptomatic patients compared with controls. It is unlikely that the ring staining represented IL-4 bound to its receptor (IL-4R) as many cell types express the IL-4R and might also have been expected to stain but were not observed to do so. The possibility that the ring staining represented degraded IL-4 product was also unlikely as the proportion of cells positive for both types of staining was small, and the proportion of 3H4 positive cells rose in the absence of a significant rise in 4D9 positive cells. In addition this pattern of staining was observed in isolated cells [Bradding et al.1995a] suggesting that this epitope may have been associated with mast cell membrane glycoproteins rather than represent IL-4 product degraded in the immediate extracellular environment. The numbers of cytoplasmic 4D9$^+$ cells fell in symptomatic patients compared with numbers in both asymptomatic and normal controls is consistent with cytokine release during degranulation. Finally, the high proportion of MC's observed to

express IL-4 mRNA with little variation between patient groups suggested that cytokine storage and release from mast cell granules, rather than induced transcription of IL-4 mRNA, may play an important role in the regulation of IL-4 activity.

The actions of IL-4 are critical in promoting allergic reactions. IL-4 and recently, IL-13 have been shown to be capable of providing the signal stimulating B cells to isotype switch from IgG1 to IgE and IgG4 production [de Vries et al. 1995]. IL-4 promotes T cell growth, directs the development of T_H2-like cells and upregulates the expression of the low affinity IgE receptor FcεRII (CD23) and MHC class II molecules on monocytes and B cells [Swain et al. 1990]. Transgenic mice in whom the IL-4 gene is over-expressed develop a severe blepharo-conjunctivitis characterized histologically by mast cell and eosinophil accumulation [Tepper et al. 1990]. In contrast, knockout mice with an IL-4 gene deletion are unable to mount IgE responses [Kuhn et al. 1991]. Although human T cells are known to be an important source of IL-4, they themselves require a pulse of IL-4 for STAT-6 activation in order to induce and maintain GATA3 [Rao et al.2000] which ensures the commitment of the T_H2 lineage. T cells have not been demonstrated to store significant quantities of this cytokine in their cytoplasm using immunohistochemistry [Bradding et al.1992]. Mast cells may also contribute to IgE regulation through direct cell to cell contact with B cells. Mast cells are capable of stimulating B cells to isotype switch via a CD40 / CD40 ligand mechanism in the presence of IL-4 independently of T cell help [Gauchat et al.1993]. The findings from this study suggest that the mast cell is an important source of IL-4, particularly during the early phase of the allergic response, and that this capacity to release IL-4 may contribute significantly to the upregulation of this response. Interestingly, histamine has recently been demonstrated to enhance IgE and IgG4 production in purified B cells stimulated with either IL-4 or IL-13 in the presence of anti-CD58 mAb [Kimata et al. 1996]. These cytokines have also been demonstrated to induce histamine release from basophils [Kimata et al.1996] raising the possibility that in atopic patients IL-4, IL-13 and histamine may form a vicious circle leading to disordered IgE regulation.

The recruitment of leukocytes to areas of allergic inflammation is mediated by the selective engagement of adhesion proteins. The selectin family of molecules are involved in the initial attachment phase of cell adhesion and P-selectin can be rapidly mobilized from its stores within endothelial cells to mediate the adhesion and rolling of neutrophils,

eosinophils and lymphocyte sub-types. IL-4 has recently been shown to induce prolonged expression of P-selectin on human endothelial cells [Yao et al. 1996] although the significance of this in SAC remains to be determined. The cell marker study (Section 5.1.1) found low numbers of neutrophils and eosinophils in normal conjunctiva with no significant increase in cell numbers during symptomatic disease. At higher pollen levels than the subjects experienced however, these cells may play a more important role since raised numbers of eosinophils and neutrophils have been detected in conjunctival scrapings following high dose allergen challenge [Bonini et al.1988]. Members of the immunoglobulin superfamily, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) have also been shown to be regulated by IL-4 which downregulates ICAM-1 expression and upregulates VCAM-1 expression. This mechanism may lead to the selective recruitment of eosinophils which express very late antigen-4 (VLA-4), a ligand for VCAM-1. A more powerful inducer of ICAM-1 and VCAM-1 expression however, is TNFα. Mast cells have been shown to be an important source of this cytokine and are capable of releasing significant preformed stores of TNFα as well as newly synthesized product [Gordon et al.1990a]. Mast cells appear to be the principal regulators of adhesion proteins through TNFα production [Meng et al. 1995] and this process would provide an important mechanism for the sustained T cell recruitment observed in Chapter 5. In this study mast cells were noted to be the principal source of preformed TNFα in the conjunctiva although the proportion of TNFα containing cells fell in SAC in and out of season. Only a small proportion of mast cells appeared to contain TNFα with the majority of biopsies in all groups showing no TNFα positive cells. This is in contrast to upper respiratory mucosa in which 45% of mast cells were found to contain TNFα [Bradding et al.1995a]. Several factors may have contributed to this difference. Nasal mucosa is continuous with conjunctival mucosa through the lacrimal passages but the two tissues have very different functions. The nasal passages filter the environmental air and as such, this mucosa would be expected to come into contact with a variety of irritants e.g. air pollutants, and potential pathogens which might be expected to result in neutrophil and eosinophil recruitment. Although directly exposed to the environment, the conjunctiva has no analogous function and the accumulation and activation of these cells during SAC would be extremely deleterious to the cornea (see section 5.1.1). Lastly, the method of detection used in this study was probably more specific but less sensitive than

that used to detect TNFα positive mast cells, this would also have contributed to the lower counts obtained.

Mast cells were observed to be a significant source of IL-6, but not of IL-8 or IL-5 although IL-5 and IL-6 cell counts were low in all groups and the median IL-8 count was zero including symptomatic patients. In addition, IL-5 was not co-localized to eosinophils in any group. No significant changes in the numbers of IL-5 or IL-8 positive cells were observed in SAC either in the symptomatic or asymptomatic subjects. IL-5 and the C-X-C chemokine, IL-8, are potent eosinophil and neutrophil chemoattractants [Shute 1994; Wang et al. 1989] neither of which were observed in increased numbers in the SAC patients. These findings support the observation that eosinophil and neutrophil numbers did not rise in symptomatic subjects with SAC compared with asymptomatic subjects or controls (Chapter 5). The recruitment of eosinophils is characteristic of VKC and AKC but not SAC where corneal epithelial damage from eosinophil cationic proteins is not seen.

In summary, this study has shown that conjunctival mast cells are an important source of multifunctional cytokines. Mast cells are traditionally recognized as the central effector cells of acute hypersensitivity but they are also ideally positioned to orchestrate the early immune response to allergen prior to leukocyte recruitment and activation. The finding that the large majority of IL-4 positive cells were mast cells and that this increased significantly in symptomatic SAC suggests that the mast cell may play a significant role in IgE regulation through both cell-mediated and humoral mechanisms.

CHAPTER 7

The relative contribution of MC_T and MC_{TC} mast cell subsets to conjunctival T_H2-like cytokines

7.1 Introduction

Mast cells are recognized as central effector cells in atopic diseases including allergic conjunctivitis. In particular, mast cells are known to play important roles in the pathology of seasonal allergic conjunctivitis (SAC), a mild, prevalent but poorly understood form of ocular allergy [Anderson et al. 1997a]. During active SAC, conjunctival mast cell numbers have been shown to increase [Anderson et al. 1997b] and elevated levels of histamine, tryptase and leukotriene C_4 have also been detected in tears [Abelson et al.1980; Bisgaard et al.1985; Butrus et al.1990]. It is now known that mast cells, in addition to T cells, are a source of pleiotropic cytokines known to play key roles in the regulation of allergic diseases [Plaut et al.1989]. Interleukin (IL)-4, IL-5, IL-6 and IL-13 have been localized to mast cells at both protein and mRNA level [Bradding et al.1992; Bradding et al.1993; Bradding et al.1995a; Burd et al. 1995; Moller et al.1993; Okayama et al.1995] and human conjunctival mast cells have also been shown to be a source of cytokines [MacLeod et al. 1997]. Mast cells represent a heterogeneous population of cells however, which vary according to their tissue distribution, their response to immune and non-immune stimuli and their biochemical content. In humans, two subsets are described based on their content of neutral proteases; those containing tryptase, chymase, carboxypeptidase A and cathepsin G which are termed MC_{TC}, and those containing tryptase only, termed MC_T [Irani et al.1986]. Although differences between mast cells located at different sites have been documented (see review [Irani et al.1989]), little is understood about the functional differences between subsets within a given tissue.

In the present study, we sought to investigate the normal pattern of cytokine expression in MC_T and MC_{TC} subsets. To explore changes during 'natural' allergen exposure, we studied patients with SAC at a single time point when they were symptomatic or

asymptomatic with itch, and compared them with normal controls. In addition, we sought evidence that conjunctival mast cells are capable of sustained IL-4 production through the synthesis of IL-4 mRNA.

7.2 Materials and Methods

7.2.1 Immunohistochemistry

This was performed as previously described (Chapter 4). The following mouse IgG_1 monoclonal antibodies were used at previously titrated optimal dilutions: AA1 to tryptase (Dr A.F.Walls, Immunopharmacology, Southampton University, UK.), MAB1254 to chymase (Chemicon, Harrow, UK.), 3H4 and 4D9 to IL-4 (Dr C Heusser, Ciba-Geigy, Switzerland), MAB7 to IL5 (Dr L McNamee, Glaxo, UK.), 104B11 to IL6 (Dr P Hissey, Glaxo, UK.), MAB 213 to IL13 (R&D Systems Ltd, Oxford, UK.), UCHT1 to CD3 (Dako Ltd, High Wycombe, UK.). Control slides were treated identically, one with omission of the primary antibody, the other with a non-specific IgG_1 monoclonal antibody at the highest concentration of primary antibody used (M9269 Sigma, Poole, UK.).

7.2.2 Quantification and statistical analysis

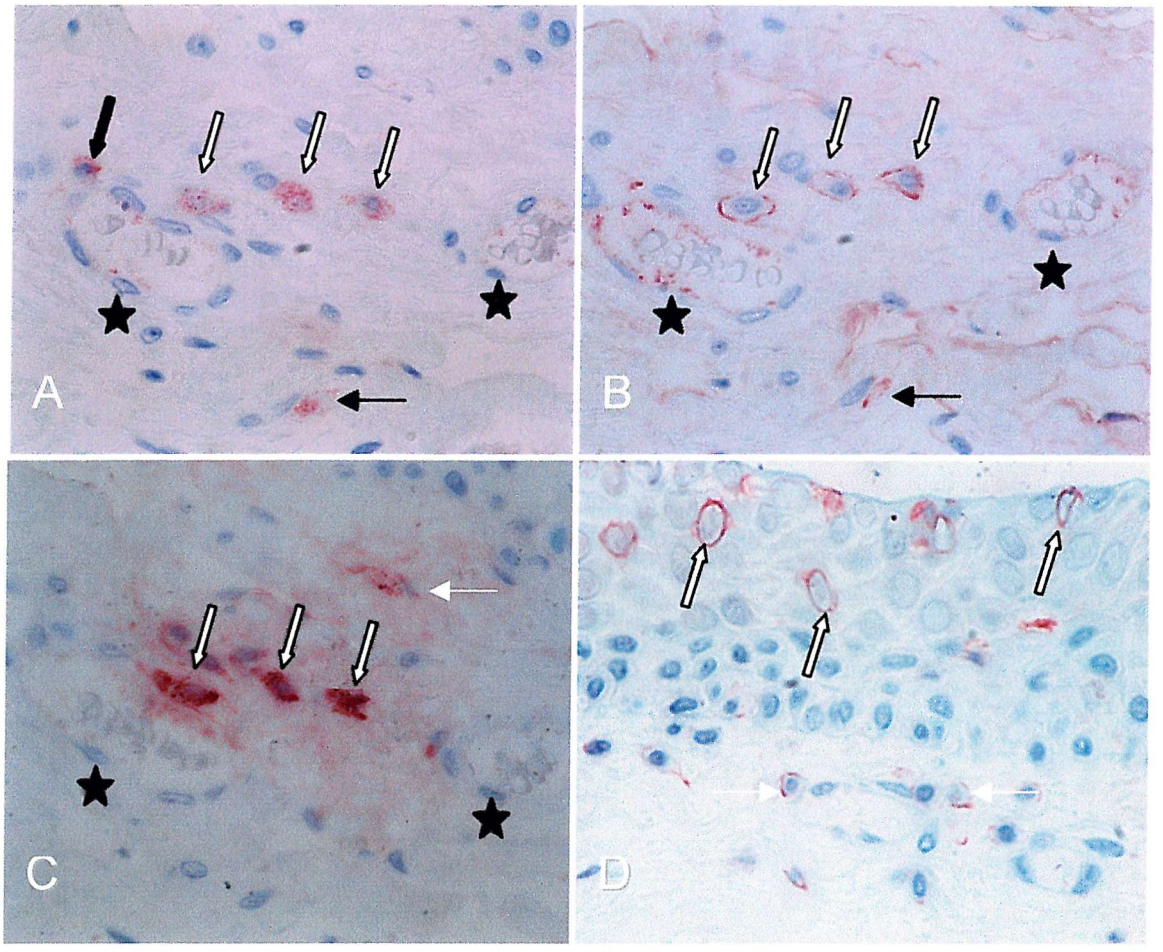
Cells were counted and data described and analyzed as previously described in Section 4.

7.3 Results

Clear immunohistochemical staining for IL-4, IL-5, IL-6, IL-13, mast cell tryptase and chymase and $CD3^+$ T cells was observed with no staining in the control slides. Examination of sequential sections enabled the identification of the same cells to be performed using morphological landmarks and the co-localization of cytokine product to mast cell subtypes (Figure 6.2, Chapter 6). In some cases, immunoreactivity of positive granules could not be co-localized to nucleated cells (Figure 7.1) and these were not counted. The mAb 3H4 to IL-4 gave a predominantly ring staining pattern (Figure 7.1) although cytoplasmic staining was observed in a significant proportion of cells. This

FIGURE 7.1

Composite images to show co-localization of mast cell IL-4 with tryptase and chymase positive cells



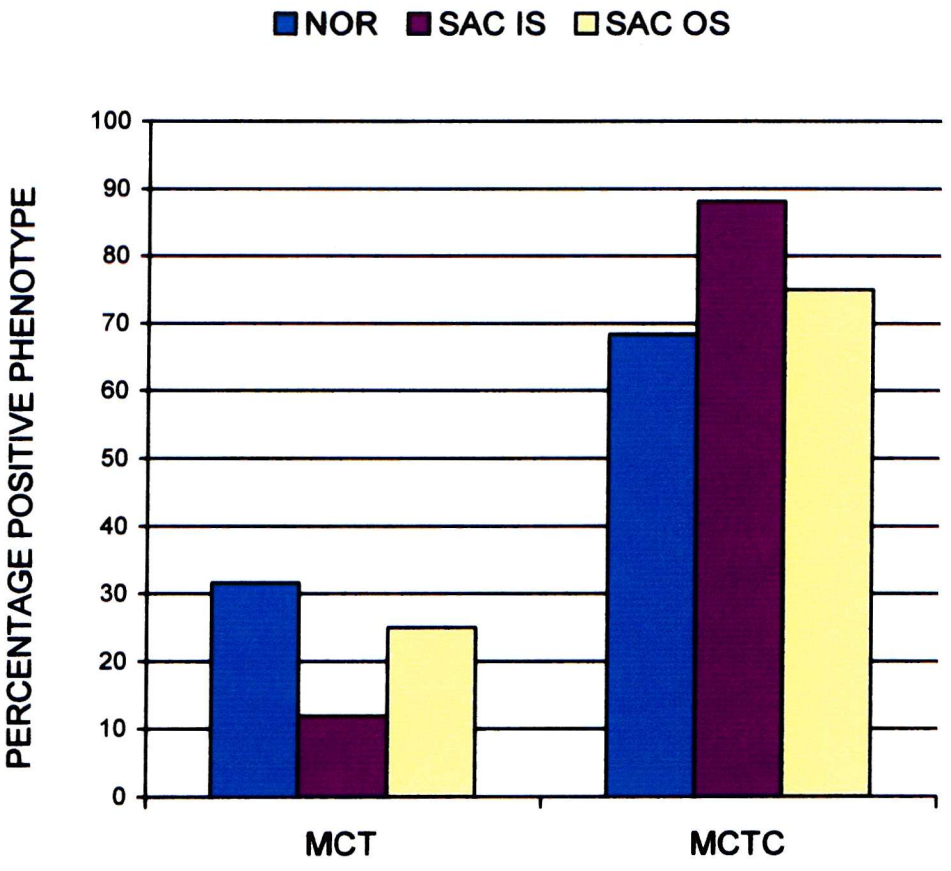
Legend

Figure 1A to 1C shows sequential 2μm sections through the same biopsy orientated for examination by using blood vessels (black stars) as morphological landmarks. Mast cell tryptase was seen as granular cytoplasmic staining (white block arrows) (1A). These arrows highlight the same cells in the adjacent sections but labeled for IL-4 (1B) which appeared as a ring staining pattern using the mAb 3H4, and labeled for mast cell chymase, which was seen as granular cytoplasmic staining (1C). Black block arrows show a tryptase positive cell in (1A) that did not co-localize with either IL-4 or chymase. A fine black arrow shows a tryptase positive mast cell that co-localized with IL-4 but not chymase (1A) and (1B). When granular staining alone was present, but no nucleus was visualized, the cell was neither co-localized nor counted (white fine arrow)(1C). T cells were clearly visible within the epithelium (white block arrows) and the substantia propria (fine white arrows) using a mAb to CD3 (1D).

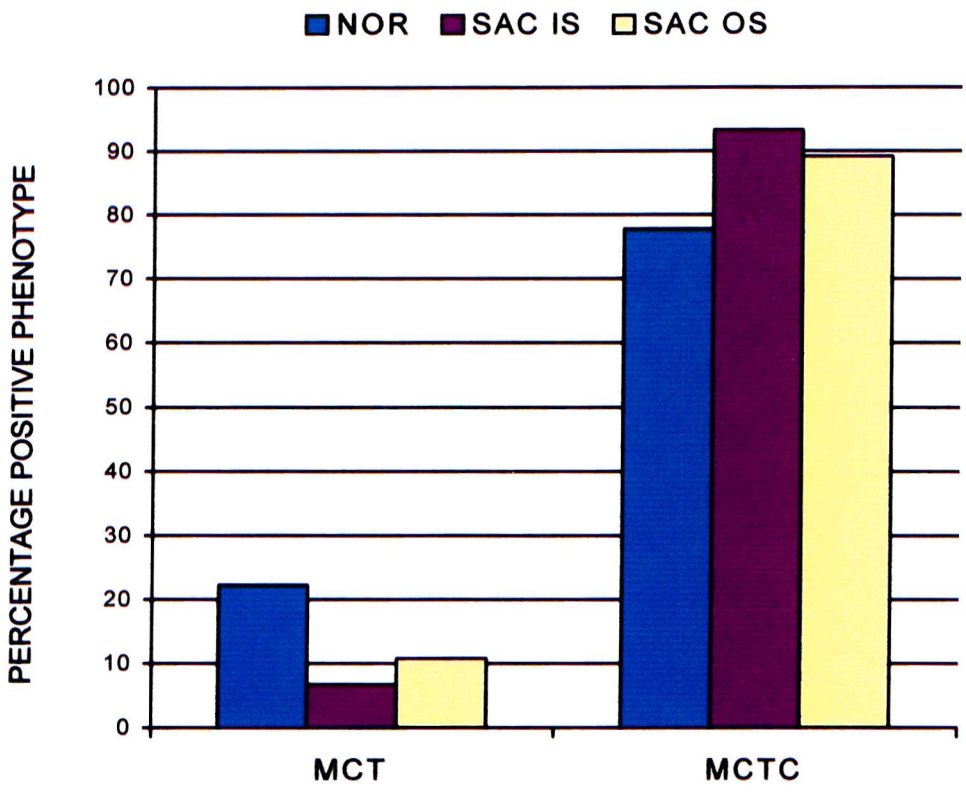
FIGURE 7.2

Distribution of mast cell phenotype between the total population of mast cells that were immunopositive for IL-4 using two different labels

A) Cell surface IL-4 using mAb 3H4



B) Cytoplasmic IL-4 using mAb 4D9



Legend

NOR, normal controls; SAC IS, symptomatic SAC subjects in season; SAC OS, asymptomatic SAC subjects out of season. The total proportion of MC_{TC} (tryptase$^+$ chymase$^+$ cells) and MC_T (tryptase$^+$) cells in each case was 100%.

pattern had previously been imaged by double immunofluorescence confocal microscopy which clearly demonstrated $3H4^+$ staining at the mast cell surface (Chapter 6 Figure 6.10). Staining with the mAb 4D9 to IL-4 gave predominantly cytoplasmic staining although a few ring staining cells were noted. Immunostaining for IL-5, IL-6 and IL-13 was cytoplasmic with very few cells displaying surface staining. Mast cells were observed to stain for both 3H4 or 4D9 mAb's in sequential sections. Although $3H4^+$ mast cells were often positive for both 3H4 and 4D9 epitopes of IL-4, this was not always the case. $4D9^+$ mast cells were not always $3H4^+$, although the numbers in each group were too small to achieve a statistically significant comparison. No $CD3^+$ cells were noted to co-localize with either 3H4 or 4D9 immunoreactivity. Mast cells were predominantly located sub-epithelially and around blood vessels with no mast cells noted in the epithelium in control biopsies or from asymptomatic subjects. Two intra-epithelial mast cells were observed in two symptomatic SAC patients. Although the large majority of $chymase^+$ cells were also $tryptase^+$, some $chymase^+$ $cytokine^+$ only cells were observed.

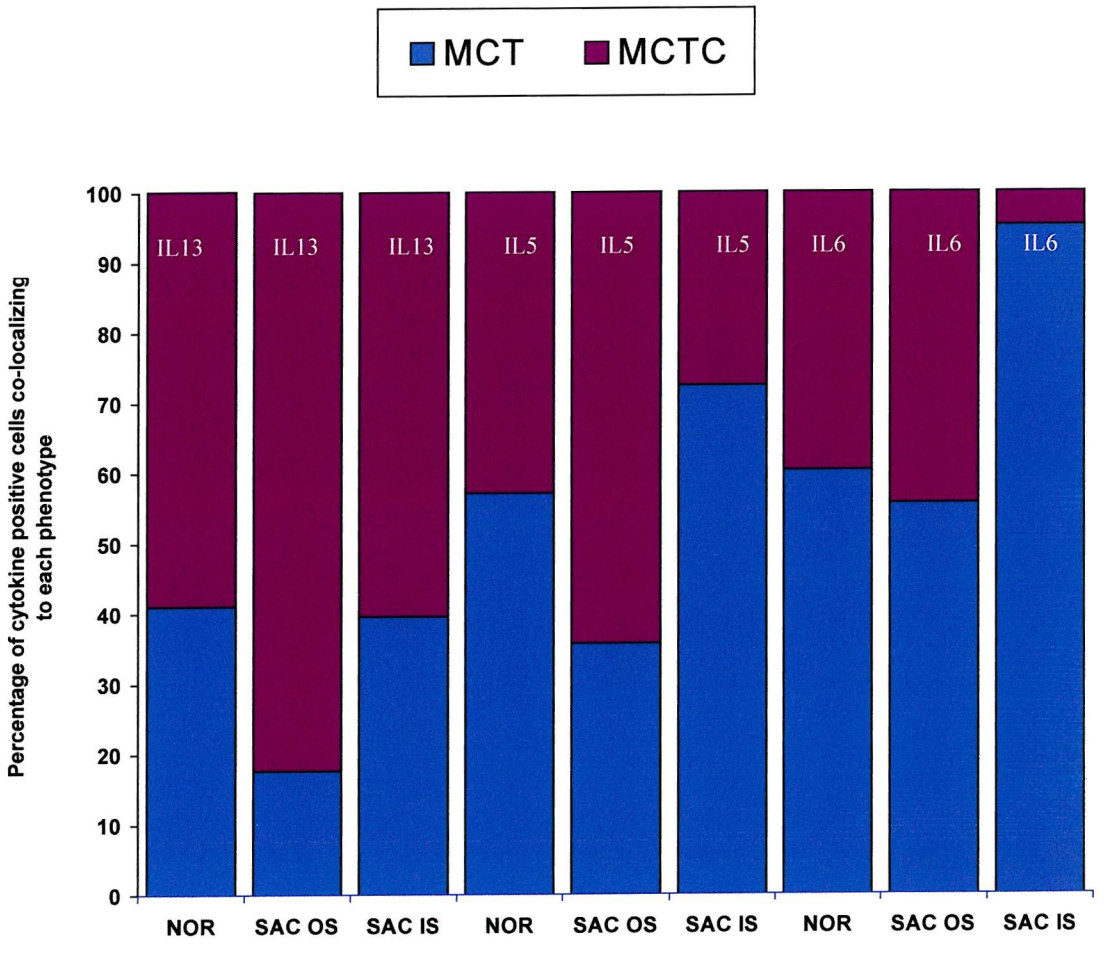
7.3.1 Mast cell heterogeneity

In all patient groups, the majority of mast cells were of MC_{TC} phenotype, with the greatest difference noted in symptomatic SAC subjects (79.1% MC_{TC} vs. 20.9% MC_T, p= 0.0008), this difference was less marked although significant in asymptomatic SAC (65.1% MC_{TC} vs. 34.9% MC_T, p= 0.015), and normal (% 71.5 MC_{TC} vs. 28.5% MC_T, p= 0.02) subjects.

IL-4^+ mast cells displayed clear phenotypic heterogeneity; ring $3H4^+$ immunoreactivity co-localized significantly to MC_{TC} cells in symptomatic (p=0.0008) and asymptomatic (p=0.006) SAC subjects (Figure 7.2A) whilst $4D9^+$ immunoreactivity co-localized significantly to MC_{TC} cells in all groups (normals p=0.0472, symptomatic SAC subjects p=0.0017 and asymptomatic SAC subjects p=0.0008)(Figure 7.2B). A less consistent pattern was observed for IL-13^+ mast cells with the greatest difference noted in asymptomatic SAC subjects (Figure 7.3). Interleukin-5^+ and IL-6^+ immunoreactivity was

FIGURE 7.3

The distribution of mast cell phenotype between the total population of mast cells which were immunopositive for IL-5, IL-6 and IL-13



Legend

Distribution of mast cell phenotypes by protease expression among mast cells reactive for cytoplasmic IL-5, IL-6 and IL-13. Bars represent the distribution of cytokine positive cells between MC_T and MC_{TC} cells for each patient group.

variable but appeared to preferentially co-localize to MC_T cells except for IL-5^+ mast cells in asymptomatic SAC subjects (Figure 7.3), the differences in distribution did not achieve statistical significance.

7.4 Discussion

Evidence for the immunolocalization of a panel of cytokines to mast cells was presented in Chapter 6. The results obtained in this study demonstrate that the conjunctival mast cell population displays significant heterogeneity in the pattern of T_H2-like cytokines distributed between human mast cell subsets. In particular, IL-4 and IL-13, known to drive T_H2-like allergic responses, were observed to preferentially immunolocalize to the MC_{TC} phenotype suggesting that this subset of mast cells may play functionally different roles to MC_T cells in atopic reactions.

Circulating mast cell precursors have not been identified, and it is not known whether increases in tissue numbers reflect an expansion of existing cells, recruitment of $CD34^+$ precursors from the bone marrow or a mixture of the two. The recruitment of pro-inflammatory cells including T cells takes place in part through direct chemotaxis, and partly through the selective upregulation of adhesion molecules which bind to T cell and granulocyte ligands and enable them to home to areas of inflammation (Chapter 1). The expression of the adhesion proteins ICAM-1 (CD54) and VCAM-1 (CD106) whose ligands are found on T cells and granulocytes, in conjunctival biopsies taken from patients with allergic conjunctivitis was recently demonstrated [Bacon et al. 1998]. These data are consistent with the above finding of a striking increase in T cell numbers both in the epithelium and substantia propria of conjunctiva from symptomatic compared with asymptomatic patients.

Mast cells display marked heterogeneity with respect to distribution, mediator content, phenotype and activation characteristics [Irani et al.1989]. We observed a significant majority of MC_{TC} cells in conjunctiva from symptomatic and asymptomatic SAC subjects as well as normal controls. This observation was in contrast to the previous finding of increased numbers of MC_T in normal conjunctiva with an increase in MC_{TC} in conjunctiva

from subjects with SAC [Baddeley et al.1995]. Although we took larger numbers of biopsies from normal subjects with a similar age range distribution to our previous report, there were several potential reasons for the observed difference. 1) Inflammatory cell numbers in normal conjunctiva are known to be highly variable, a finding we observed within the same biopsy and between biopsies using specific immunohistochemistry, and reported by Allansmith et al. over twenty years ago using tinctorial methods of cell identification [Allansmith et al.1978]. 2) We may have previously underestimated the number of MC_{TC} cells; by co-localizing cells according to the presence of a nucleus in one adjacent section only, we were likely to have increased the number of cells counted compared to the number previously recorded which required the detection of a nucleated cell in both sequential sections. 3) The protease phenotype of the mast cells may not have remained stable within a given conjunctiva over time; although mast cells are known to be capable of regranulation and continued responsiveness to stimuli, it is not known whether the protease phenotype remains the same. A few mast cells were observed to be positive for chymase only, and chymase$^+$ cytokine$^+$ mast cells in the absence of tryptase positivity. This interesting observation may have been due to the deficiency of the co-localizing technique or may have been a true difference, as chymase only positive mast cells have been obtained in culture using stem cell factor [Li et al. 1996b].

In addition to protease heterogeneity, IL-4 and IL-13 were observed to co-localize preferentially to the MC_{TC} subset in disease and control groups. Interleukin-4 is well recognized to upregulate humoral, and suppress cell-mediated immunity by stimulating the differentiation of the T_H2 subset of T helper cell [Swain et al.1990] known to produce IL-4, IL-5, IL-6 and IL-13 [Ryan 1997]. Although T cells may stimulate B cell IgE production, mast cells are also able to induce B cell isotype switching to IgE synthesis through direct CD40/CD40 ligand binding and through the release of IL-4 and IL-13 independently of T cell help [Pawankar et al. 1997]. In turn, IL-4 has also been shown to upregulate the high affinity IgE receptor component, FcεRIα chain, on human mast cells [Toru et al.1996]. Interleukin-5 and IL-6 appeared to co-localize to the MC_T subset although the difference in distribution was less marked and IL-5 and IL-6 cell counts were low in all groups including symptomatic patients. This finding was consistent with the previous findings in Chapter 6 and with the observation of increased mast cell but not eosinophil numbers during symptomatic SAC (Chapter 5).

The linkage of markers at or near the IL-4 gene in the 5q31.1 region of chromosome 5 with serum IgE concentration suggests that the IL-4 gene or a nearby gene may be responsible for overall IgE regulation [Marsh et al.1994]. Although the genes for IL-4, IL-13 and IL-5 are closely spatially related, the finding that these cytokines were not grouped together in one subtype of cell suggested that they may be controlled separately although expressed together under certain conditions.

The preferential distribution of T_H2-like cytokine immunoreactivity between MC_{TC} and MC_T cells observed in this study supports the hypothesis that these subsets may play different roles during physiological or pathological conditions. The finding that some mast cells were positive for IL-4 alone, whilst others were positive for more than one cytokine has been observed in airway submucosa [Bradding et al.1993] and corroborates the results of this present study. Both 3H4 and 4D9 mAb's to IL-4 displayed a significant preferential co-localization to the MC_{TC} subset. The proportion of $3H4^+$ mast cells was noted to rise significantly between symptomatic and asymptomatic SAC patients whilst the proportion of $4D9^+$ mast cells was noted to fall in symptomatic patients compared with numbers in both asymptomatic and normal controls. This observation may have arisen through sampling error, or may have occurred secondary to a true fall in numbers. As the population of patients from whom the biopsies were taken for both studies overlapped, the sensitivity of the second method of co-localization may have been a significant factor. A true fall in the number of $4D9^+$ mast cells would be consistent with cytokine depletion during degranulation.

The findings that mast cells were an important source of the preformed conjunctival T_H2-like cytokines IL-4, IL-13, IL-5 and IL-6 and that the distribution of these cytokines was strongly related to cell phenotype enhances our understanding of the role of the conjunctival mast cell in the local immunoregulation of SAC. As the principle source of mast cell IL-4 and IL-13, these observations provide a potential new mechanism for MC_{TC} cells to bias the conjunctiva toward the development of a T_H2-like immune response prior to IgE mediated activation. Although the concept of mast cell subsets based on cytokine secretory pattern has yet to be explored, the functional significance of this heterogeneity suggests an important role for the MC_{TC} cell in atopic and non-atopic disorders of the conjunctiva.

CHAPTER 8

Mast Cell Stem Cell Factor

8.1 Introduction

Human mast cells are a source of multifunctional cytokines [Bradding et al.1992; Bradding et al.1993; Galli et al. 1991; Gordon et al.1990b; Gordon et al.1990a; Gordon et al.1991; Walsh et al.1991]. Rodent and human mast cell growth and maturation depends on the presence of a network of cytokine growth factors [Arock et al. 1994; Rossi et al.1998; Saito et al.1996]. Strong evidence suggests that the primary regulator of mast cell growth and function is stem cell factor (SCF), the ligand for the mast cell membrane *c-kit* gene product (CD117) [Galli et al.1992; Galli et al.1994a; Tsai et al. 1991b; Tsai et al. 1991a]. This cytokine was first described as a haematopoitic growth factor, primarily derived from fibroblasts [Levi-Schaffer et al. 1991] and the surrounding tissue stroma [Galli 1990]. SCF has been demonstrated to induce mast cell growth and differentiation [Irani et al.1992; Mitsui et al. 1993; Valent et al.1992], suppress mast cell apoptosis [Iemura et al. 1994], enhance mast cell mediator [Bischoff et al.1992; Columbo et al. 1992] and differential cytokine release [Gagari et al. 1997; Okayama et al.1995], mediate mast cell adhesion to the extracellular matrix [Dastych et al.1994] and function as a mast cell chemoattractant [Meininger et al.1992]. Since many other inflammatory cells have the capacity to synthesize and secrete autocrine factors which influence their growth, function and development e.g. eosinophils (IL5, GM-CSF) and neutrophils (IL8), the aim of this work was to investigate whether human mast cells were a source of this cytokine and to explore differences, if any, in SCF immunoreactivity between MC_T and MC_{TC} subtypes.

8.2 Materials and Methods

The diagnosis of SAC was made according to the criteria previously described. Conjunctival biopsies were obtained from four symptomatic atopic patients (2 female, 2

male, mean age 41.7 years range 23-68) with SAC (SAC IS), seven asymptomatic atopic patients (2 female, 5 male, mean age 35.6 years range 19-70) with SAC out of season (SAC OS). There was no statistical difference between the groups for age or sex. Conjunctival snip biopsies were obtained as previously described (Chapter 3) and divided in two to process for IHC or ISH (Chapter 4).

8.2.1 Immunohistochemistry

This was performed as previously described (Chapter 4). The following mouse IgG_1 monoclonal antibodies were used at previously titrated optimal dilutions (see Table 4.1): AA1 to tryptase (Dr A.F.Walls, Immunopharmacology, Southampton University, UK.), MAB1254 to chymase (Chemicon, Harrow, UK.) and AB 62-100 to stem cell factor (Biosource / Genzyme, UK).

Control slides were treated identically, one with omission of the primary antibody, the other with a non-specific IgG_1 monoclonal antibody at the highest concentration of primary antibody used (M9269 Sigma, Poole, UK.). Cell counting was performed as described previously.

8.2.2 *In-situ* hybridization

Stem cell factor probe synthesis was performed commercially (Oswald DNA Services), according to chosen sequences which were unique to humans [Martin et al.1990]. Probes were labeled and checked as described in Chapter 4. In-situ hybridization was performed according to the technique developed for the small biopsies obtained, this is fully described earlier (Chapter 4), the bound probe was detected using an antidigoxigenin antibody and visualized using NBT-BCIP (Sigma). Controls included a sense probe (negative), the β-actin gene which codes for a constitutive cellular protein (positive) and RN-ase pre-treated tissue (negative). Dual ISH/IHC of SCF mRNA and mast cell tryptase$^+$ cells was performed on paraffin embedded biopsies from symptomatic patients in season (72 year male, 58 year female) and normal controls (50 and 51 year females). These experiments were repeated with freshly synthesized probe to maximize the signal strength of the ISH prior to IHC.

8.2.3 Sequential *in-situ* hybridization and immunohistochemistry (ISH/ IHC)

Sequential ISH/IHC was performed to overcome difficulties in distinguishing SCF $mRNA^+$ and $AA1^+$ cells from SCF $mRNA^-$ and $AA1^+$ cells. These arose because the signal obtained using the SCF cocktail of oligoprobes was strong enough to distinguish from background staining but obscured by the strong granular staining obtained with AA1. Sequential sections were cut at 4μm thickness and adjacent sections processed for ISH using the cocktail of oligoprobes described earlier or immunostained using the mAb's AA1 to mast cell tryptase and MAB1254 to mast cell chymase. Sections were then examined using a camera lucida (Leica UK Ltd.).

8.2.4 RT-PCR for SCF mRNA in isolated human mast cells

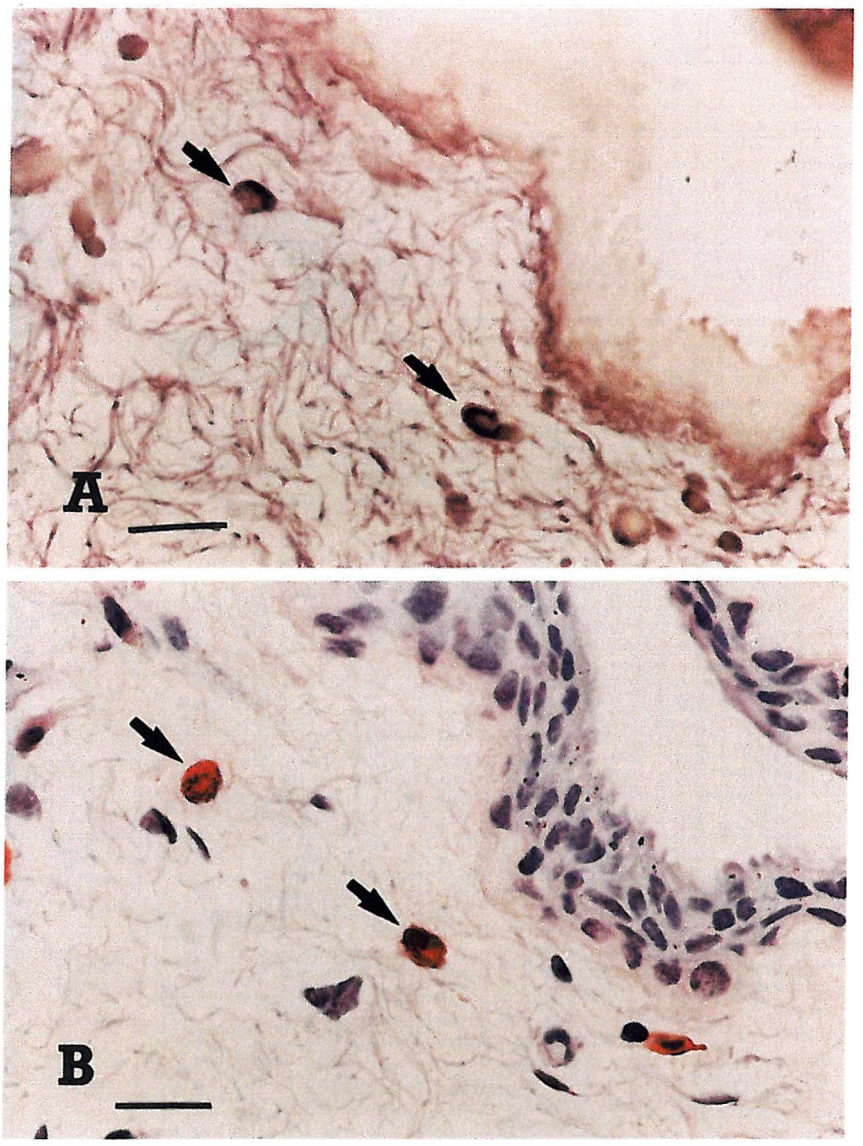
RT-PCR was performed to facilitate detection of small amounts of SCF mRNA in skin mast cells using the method described in Section 4.6.

8.3 Results

Immunoreactivity for SCF was co-localized to MC_T and MC_{TC} phenotypes in symptomatic patients with SAC in season (68.3% MC_T vs. 31.7% MC_{TC}), asymptomatic SAC patients (21.4% MC_T vs. 78.6% MC_{TC}) and normals (37.5% MC_T vs. 62.5% MC_{TC}) (p= ns). SCF mRNA expression was demonstrated in human conjunctival and skin mast cells by *in-situ* hybridization and using RT-PCR with the total cellular RNA. Although the morphology of the tissue was less well demonstrated using ISH compared with IHC, SCF mRNA could be clearly colocalized with mast cell tryptase in sequential sections (Figure 8.1). Using this technique results were qualitative as counting the proportion of SCF mRNA positive cells which were tryptase positive was not accurate or possible for the sample size of the biopsy. Control hybridizations using either sense probes or antisense probes on RN-ase treated sections were negative. Dual ISH/IHC was unsuccessful at demonstrating the simultaneous presence of message and product for SCF despite repeating the experiments and using freshly synthesized oligoprobes. In each case a weakly positive signal from the NBT-BCIP chromogen was masked by the strong staining

FIGURE 8.1

Photomicrographs of sequential *in-situ* hybridization and immunohistochemistry for stem cell factor

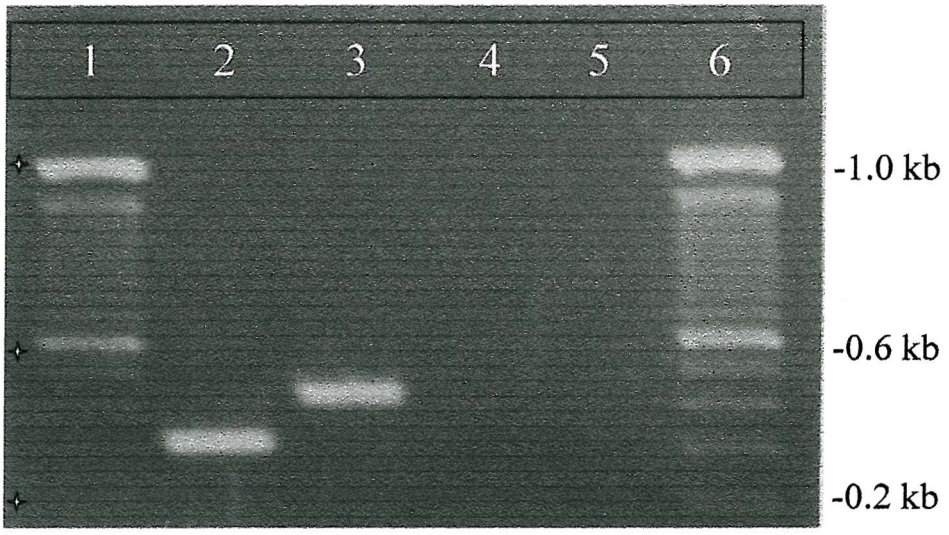


Legend

Sequential photomicrographs showing A) cells positive for SCF mRNA^{+} cells (black arrows) and in the adjacent sequential section B) the same cells positive for the mAb AA1 (black arrows). Bar is 25μm.

FIGURE 8.2

Electrophoresis of PCR product for SCF mRNA in isolated human skin mast cells



Legend

Lanes 1 and 6: DNA molecular weight markers; Lane 2: PCR with the set 1 primers (aligned at 61-90 and 342-371) amplifying a 311 bp fragment; Lane 3: PCR with the set 2 primers (aligned at 342-371 and 742-771) amplifying a 430 bp fragment; Lanes 4 and 5; negative control of PCR with two sets of primers respectively without cDNA template. Molecular weight ladder marked (with white stars) from the bottom to top at 0.2kb, 0.6kb and 1.0kb respectively.

of the AEC positive mast cell granules. Using RT-PCR with the total cellular RNA, SCF mRNA was demonstrated in freshly isolated normal skin mast cell (Figure 2). PCR-amplified SCF cDNA fragments, by two sets of primers (lane 2 and 3), were demonstrated by gel electrophoresis. In set 1 of the primers (Figure 8.2, lane 3) one of the oligonucleotides was aligned at 742-771 which recognizes the potential deletion of exon 6. This experiment thus demonstrated that normal human mast cells constitutively synthesize full length SCF mRNA.

8.4 Discussion

This work is the first to demonstrate that human mast cells express immunoreactivity for SCF and constitutively synthesize SCF mRNA. As the primary regulator for mast cell growth, survival and function, this study demonstrates that as well as being a target for SCF, the mast cell is also a cellular source for this cytokine. Although human mast cells are recognized to be a source of IL-4, IL-5, IL-6, IL-8, IL-13 and TNFα [Bradding et al.1992; Bradding et al.1995a; Burd et al.1995; Gagari et al.1997; Gordon et al.1990a; Moller et al.1993; Plaut et al.1989] these findings extend the repertoire of known cytokines synthesized by the mast cell and therefore widens their biological potential. Immunoreactivity for SCF was observed in both mast cell phenotypes in all groups with no statistical difference between the proportion of each phenotype expressing SCF.

Stem cell factor is known to be expressed in two forms[Galli et al.1994a]. The full length mRNA encodes a 248-amino acid protein which is expressed as a transmembrane protein and undergoes proteolytic cleavage to yield soluble SCF. The alternatively spliced SCF mRNA lacks exon 6 and gives rise to a smaller 220-amino acid transmembrane protein which is less efficient at producing soluble SCF as it lacks the proteolytic cleavage site. One set of primers (flanking the mRNA section from 61 to 371) recognizes a segment of mRNA encoding part of the SCF extracellular domain. The other set of primers flanks a fragment (mRNA segment from 342 to 771) within exon 6 encoding the proteolytic cleavage site. With this set of primers an amplified SCF mRNA band was identified in skin mast cells suggesting that skin mast cells are likely to express full length SCF mRNA and that they are likely to secrete the soluble molecule.

Although structural cells of the extracellular matrix are well recognized to synthesize and release SCF, mast cell SCF may act in an autocrine manner to promote mast cell survival and influence mast cell function and differentiation in both normal tissue and tissue in which mast cell accumulation e.g. SAC is evident.

CHAPTER 9

Conjunctival Allergen Challenge

9.1 Introduction

Seasonal allergic conjunctivitis has traditionally been considered as a disease characterized by a series of Gell and Coombes type I hypersensitivity reactions [Coombes et al. 1975]. This view has formed the basis of allergen challenge studies of the conjunctival mucosa in cell lines, experimental animals and man [Abelson et al. 1983b; Abelson et al.1990; Aichane et al.1993; Allansmith et al. 1989a; Allansmith et al. 1989b; Church et al. 1989; Ciprandi et al.1993a; Ciprandi et al.1994; Montan et al. 1996] with relatively few studies examining the results of natural allergen exposure [Verin et al.2001]. These studies have formed one avenue of research into the pathophysiological mechanisms of SAC and have also provided an important tool to study the potential for modifying this response by therapeutic intervention [Abelson et al. 1995; Ciprandi et al. 1995b]. Studies employing the mast cell secretagogue, compound 48/80, in rat conjunctiva [Allansmith et al.1989b; Allansmith et al.1989a] demonstrated that mast cell degranulation did occur in response to this stimulus, but that after multiple exposures, the conjunctival histology of those animals treated with compound 48/80 did not differ significantly from controls. Furthermore, although there appeared to be a threshold for triggering mast cell degranulation, this differed between mast cells at the same tissue location.

Allergen challenge is a useful model for studying acute allergic reactions; it is reproducible [Aichane et al.1993], correlates with skin challenge [Abelson et al.1990] and leads to the release of the major mediators of acute allergic reactions, histamine, PGD_2 and LTC_4 [Aichane et al.1993]. Furthermore, in some cases, conjunctival allergen challenge may be more sensitive than skin challenge in detecting grass pollen allergy [Leonardi et al. 1990;Peshkin 1932]. In 1990, Bonini et al. [Bonini et al.1990] demonstrated that a significant clinical effect and cytological changes in tears could be produced 20 mins after

CAC in atopic human subjects. Although this effect was still apparent at one hour, only those subjects challenged with the highest dose of allergen exhibited the changes. At six hours, a dose-dependant LPR was evident clinically with a median clinical symptom score of 10 out of a maximum of 17. This was accompanied by a significant increase in tear lymphocytes and eosinophils. Gleich et al. [Gleich 1982] reported that the maximal LPR in cutaneous challenge developed six hours after the initial challenge and was related to the size of the initial wheal and flare response. Moreover, passive transfer experiments suggested that only IgE was required to elicit this response and removal of IgE abolished the capacity of serum to transfer both the EPR and the LPR. This evidence supported the hypothesis that a conjunctival LPR provided a link between the acute type I hypersensitivity reaction elicited by AC and clinical disease. In 1993, Bonini [Bonini et al. 1993] proposed that the pathophysiology of SAC might be multifactorial comprising type I hypersensitivity IgE mediated mast cell degranulation, non-IgE mediated mast cell activation, non-specific conjunctival hypersensitivity and the LPR.

The finding that conjunctival mast cells are a source of multifunctional cytokines provided a potential mechanism to link the early and late phase response of SAC and thus to provide a link between CAC and SAC. The aim of this work was to try to develop a safe and reproducible method of CAC for human subjects and compare the tissue changes obtained using this method with those observed during 'natural' allergen exposure.

9.2 Materials and Methods

9.2.1 Subjects

Conjunctival biopsies were obtained from 11 atopic patients with SAC in season, allergen challenged and biopsied at either 6 or 24 hours and 9 atopic patients with SAC out of season, allergen challenged and biopsied at either 6 or 24 hours. Two normal subjects were challenged with maximal dose of allergen and biopsied at 6 hours as controls (see Table 9.1 for age and gender distribution).

TABLE 9.1

Group, age, sex and symptom score of subjects studied for CAC study

Group	Biopsy Code	Age	Sex	Symptom score at baseline	Symptom score at 20 mins	Symptom score at 6 hours	Symptom score at 24 hours
OOS CH 6	71	58	M	0			
OOS CH 6	72	34	F	0			
OOS CH 6	73	43	M	0			
OOS CH 6	126	32	F	0	10	7	-
OOS CH 6	127	60	F	0	4	0	-
OOS CH 6	128	49	F	0	11	6	-
SAC CH 24	107	28	F	0	9	1	1
	108	59	M	2	7	2	1
	119	26	F	1	9	-	0
	120	26	F	1	8	-	2
	123	36	F	1	7	-	3
	124	30	F	0	5	-	1
	141	32	M	3	11	7	4
	142	45	F	4	9	4	3
	143	27	M	6	5	2	3
SAC CH6	86	44	M				
	61	7.9	M				
N CH24	118	43	M	0	4	-	0
	111	22	M	0	0	-	1
OOS CH 24	130	21	M	0	6.5	1	0
OOS CH 24	147	47	F	0	8	5	1.5
OOS CH 24	148	49	M	0	9	2	1.5
OOS CH 24	149	21	F	0	5	1	0

Legend OOS CH, SAC allergen challenged out of season with biopsy at 6 hours; OOS CH 24, SAC subject allergen challenged out of season with biopsy at 24 hours; SAC CH 6, SAC subject allergen challenged in season with biopsy at 6 hours; SAC CH 24, SAC subject allergen challenged in season with biopsy at 24 hours

9.2.2 Conjunctival Allergen Challenge Protocol

Following skin prick testing (Section 3.2) allergen challenge was performed by asking the patient to choose which eye they preferred to have challenged and placing one drop, approx. 25µl of allergen in diluent (Soluprick ALK Laboratories, Hørsholm, Denmark.) into the inferior fornix. The patient was instructed to close the eye and after a period of 5 minutes the symptoms were scored. If the challenge was negative, the next increasing strength of allergen was used in the order:

10 SQ-U in 10µl
100 SQ-U in 10µl
1000 SQ-U in 10µl

The production of a minimum symptom score of 4 was taken as the end point of CAC. This was chosen to achieve a uniformity of response although it was recognized that the variability of the individual response to CAC would result in a higher symptom score in some subjects. If patients were to be biopsied at 24 hours, they were given a proforma to record their symptom scores at 6 hours. Conjunctival biopsy was then performed as previously described (Section 3.3).

9.2.3 Photography

Clinical photographs were taken by a dedicated ophthalmic photographer (Mr. T Mole) at Southampton Eye Unit in selected cases to document the physical signs of CAC and provide a future reference scale.

9.2.4 Immunohistochemistry

This was performed on 2µm sections of GMA embedded tissue using the protocol previously described (Chapter 4) using the a panel of mAb's to IL-4 (3H4 and 4D9), IL-5, IL-6 and TNFα, the details of which are given in Table 4.1. Negative controls comprised omission of the primary antibody and an isotype IgG1 control.

9.3 Results

One patient developed wheeze immediately following allergen challenge which required treatment with intramuscular hydrocortisone, inhaled β-1 agonists (salbutamol) and inhaled steroids (betamethasone). The adverse event occurred after the second dose of CAC and following treatment the patient made a full and uneventful recovery [Anderson et al. 1996]. This event led to the adoption of nasolacrimal duct occlusion as part of the CAC protocol. All other allergen challenges were performed without adverse incidents.

All patients developed characteristic symptoms and signs of SAC immediately following CAC including itch, foreign body sensation, hyperaemia, lacrimation and conjunctival oedema. Symptom scores (Figure 9.1) were significantly elevated from baseline to 20 minutes in the SAC CH 24 ($p=0.0006$), OOS CH 6 ($p=0.049$), OOS CH 24 ($p=0.02$) groups. Between 20 minutes and 6 hours the symptom score fell significantly in the SAC CH 24 group ($p=0.009$) and the OOS CH 24 group ($p=0.03$). Between six and 24 hours there was no significant difference in symptom score within any group, and no significant difference between baseline symptom score and score at 24 hours for any group. No patient developed symptoms or signs in the contralateral eye challenged with diluent (Appendix 1).

Clinical photographs (Figure 9.2) enabled a scale of responses to be documented.

9.3.1 Immunohistochemistry

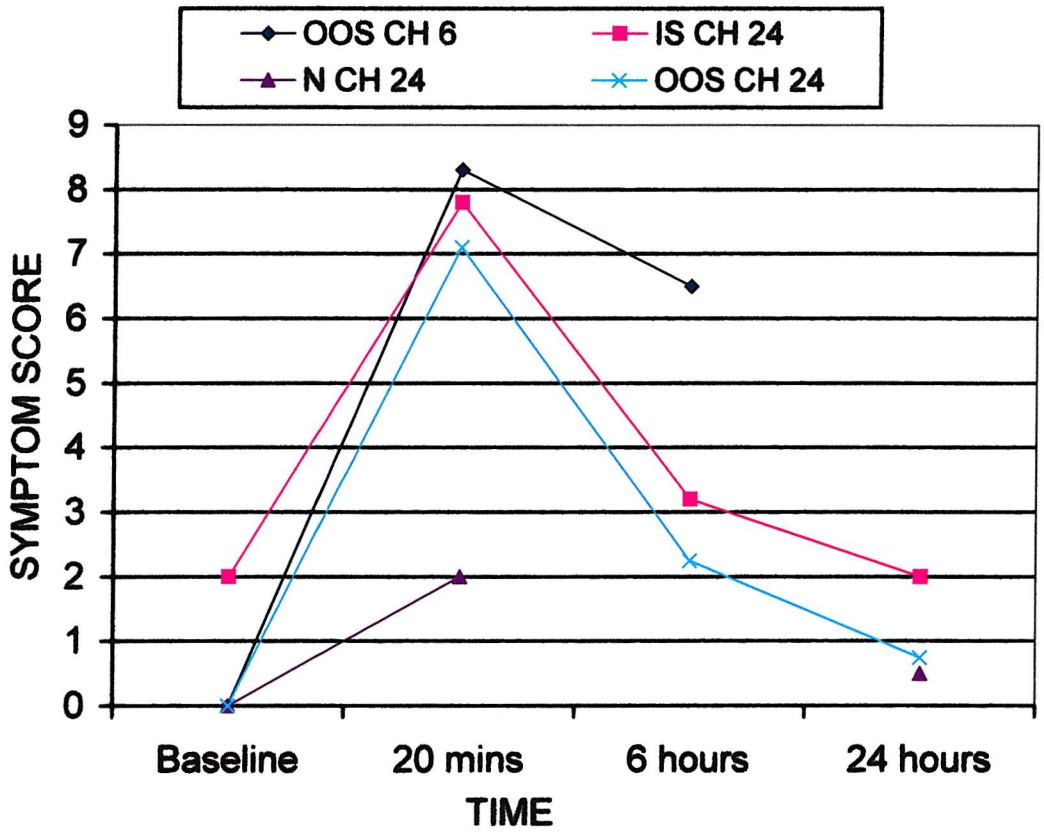
Clear staining for positively labeled cells and cytokines was evident with no staining in either omission or IgG_1 negative controls.

9.3.2 Cell and cytokine counts

The total, mean ± SEM cell and cytokine counts in cell mm^{-2} of conjunctival biopsy tissue are shown in Figure 9.3 and 9.4. There were no significant differences between mast cell, eosinophil or neutrophils numbers between symptomatic or asymptomatic SAC patients who were challenged and biopsied at 24 and 6 hours respectively, normal patients who

FIGURE 9.1

The variation of symptom score with time for each of the challenge groups and normal controls

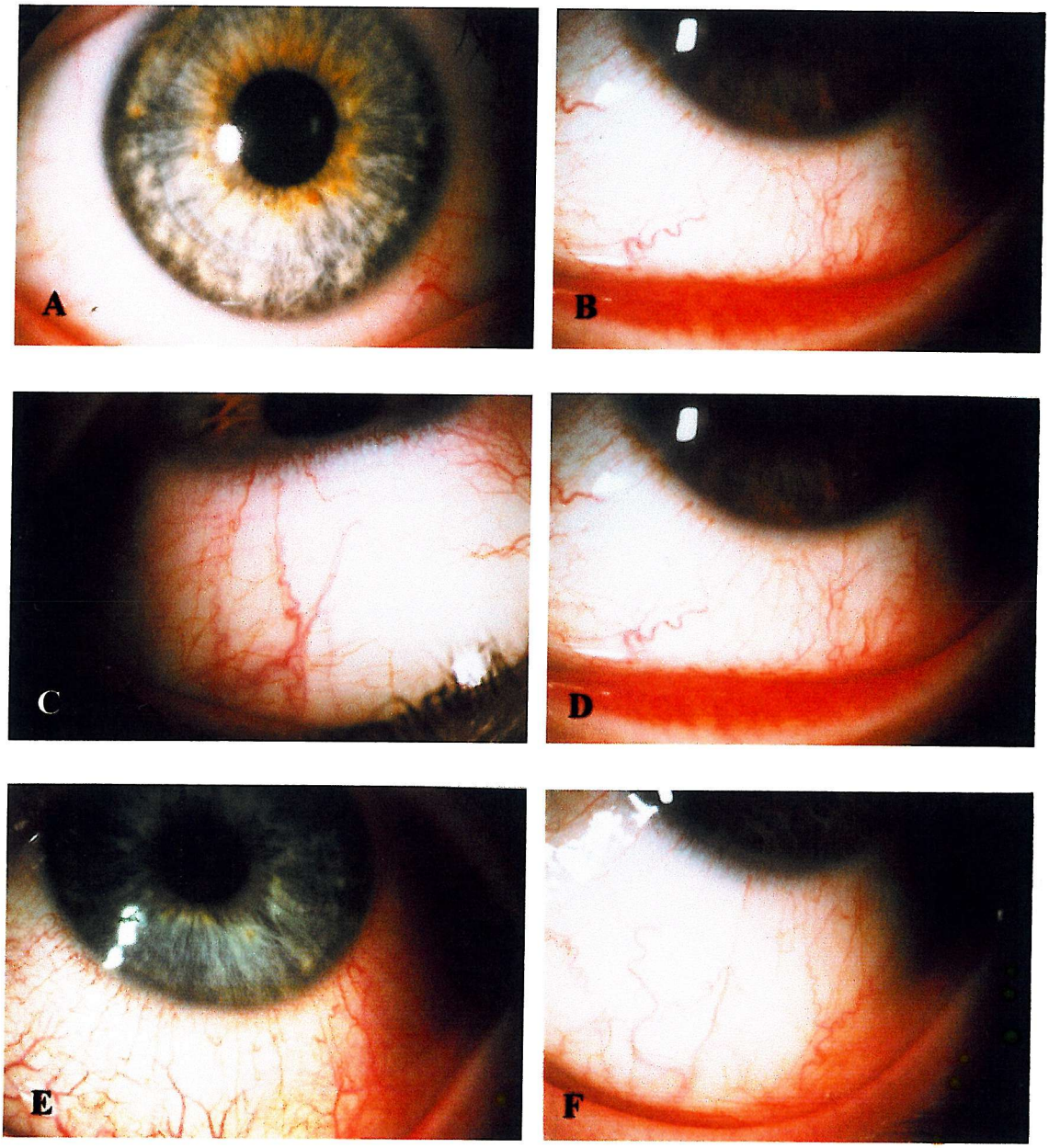


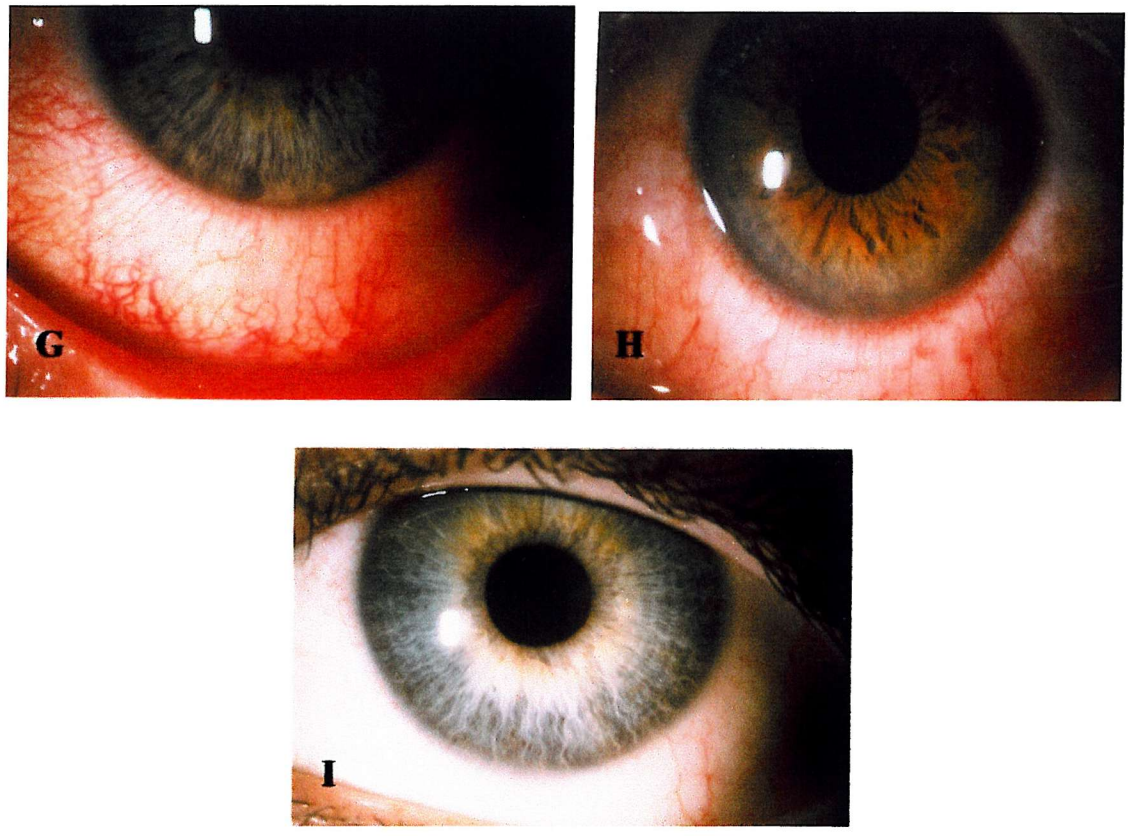
Legend

Symptom scores are the summed totals of the scores using modified Abelson [Abelson et al.1990] criteria. OOS CH 6, out of season SAC patients challenged and biopsied at 6 hours; IS CH 24, in season SAC patients challenged and biopsied at 24 hours; N CH 24, normal controls challenged and biopsied at 24 hours; OOS CH 24, out of season SAC patients challenged and biopsied at 24 hours.

FIGURE 9.2

Clinical grading photographs for conjunctival hyperaemia and chemosis



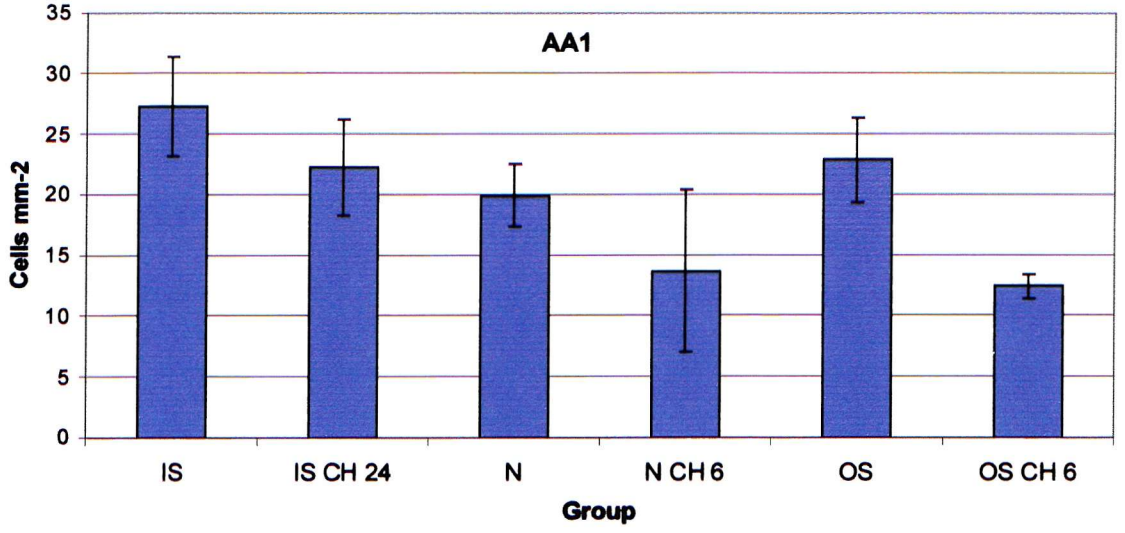


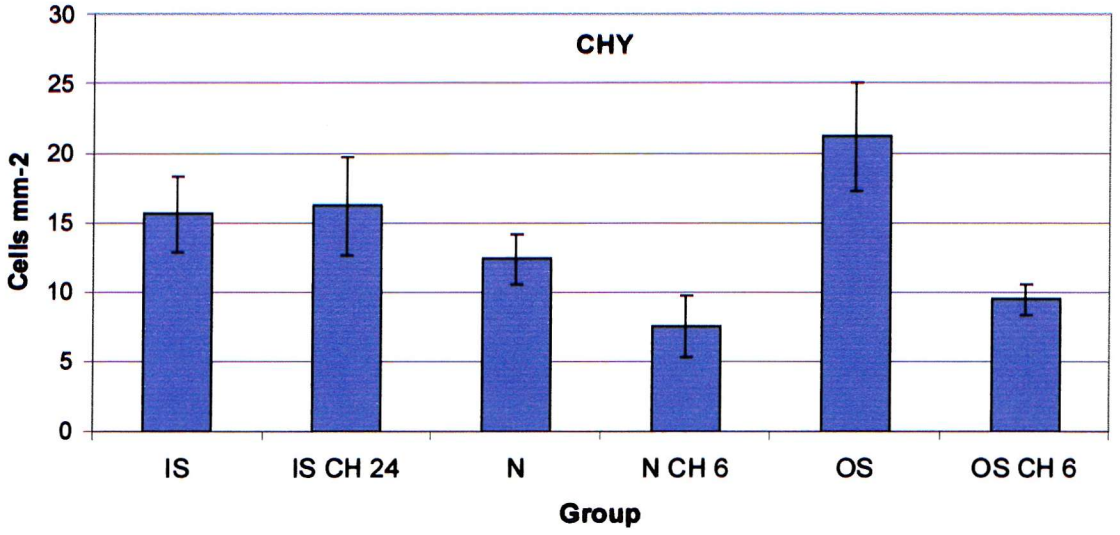
Legend

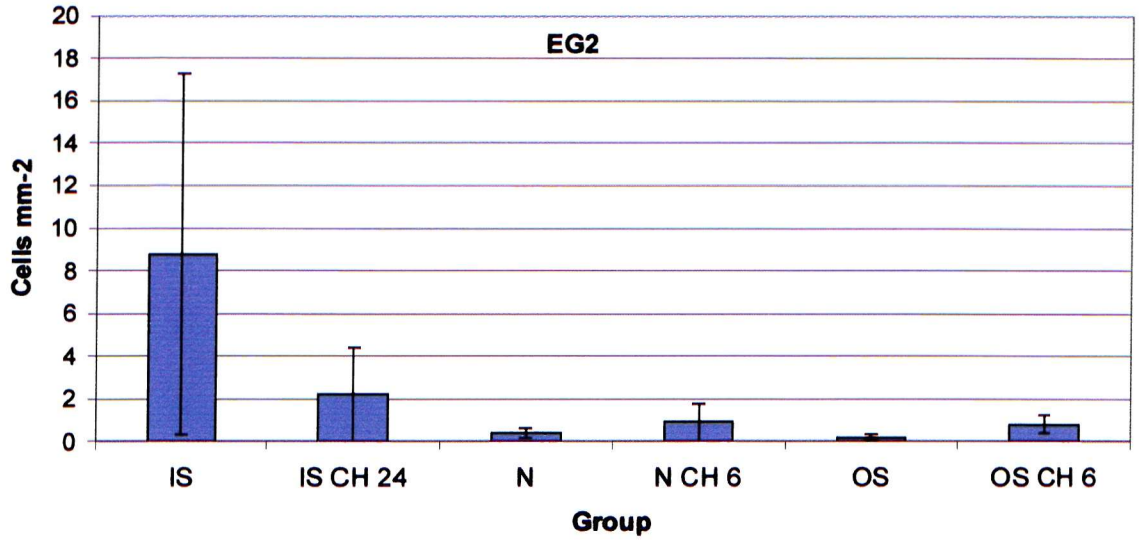
Left hand panels A, C, E and G, show increasing conjunctival hyperaemia on a scale of one to four. Right hand panels B, D, F and H, show increasing conjunctival chemosis on a scale of one to four. A control photograph of a normal conjunctiva is shown in panel I.

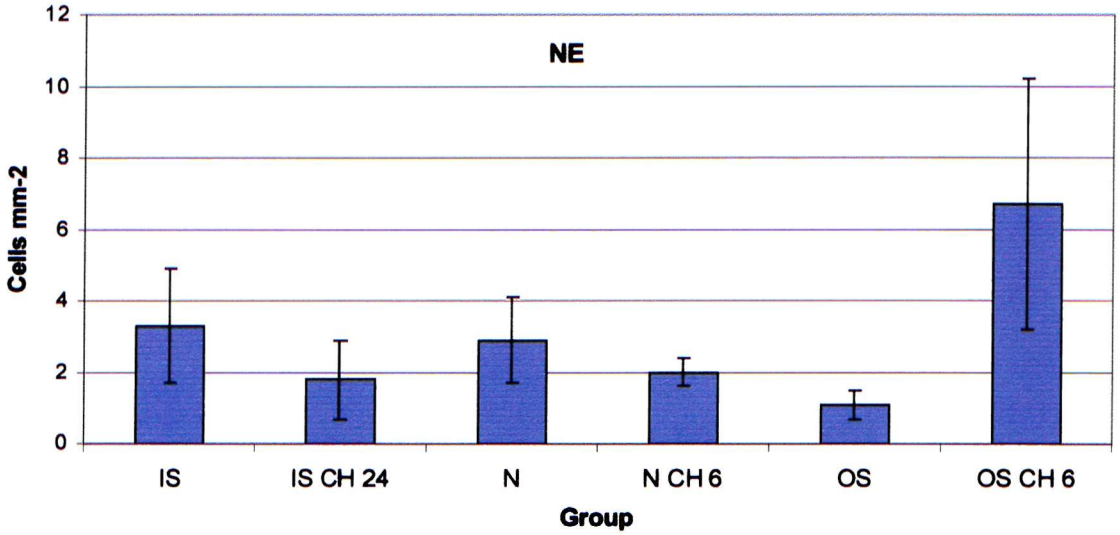
FIGURE 9.3

Mean cell counts for allergen challenged subjects, symptomatic and asymptomatic subjects with SAC and normal controls







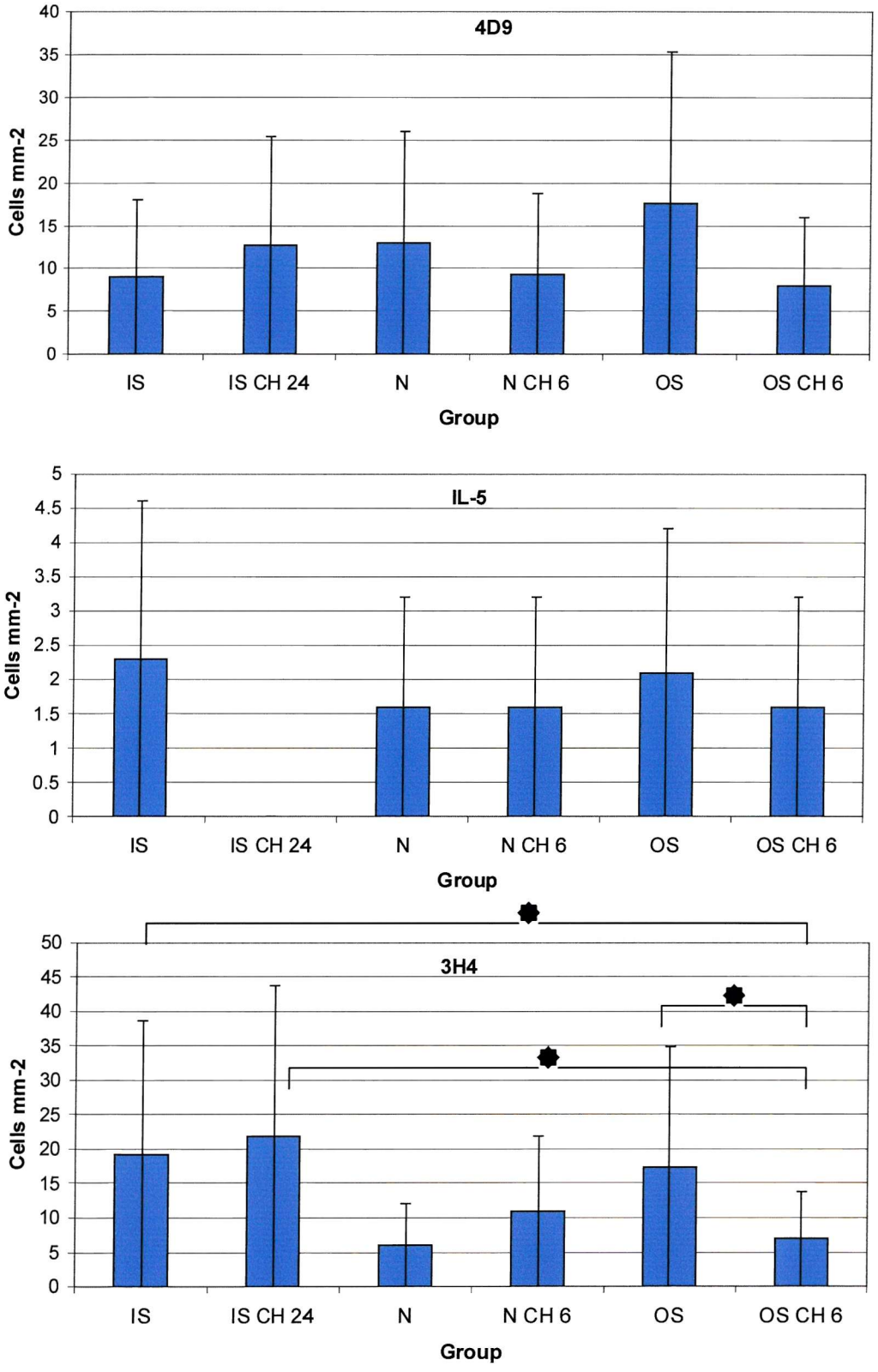


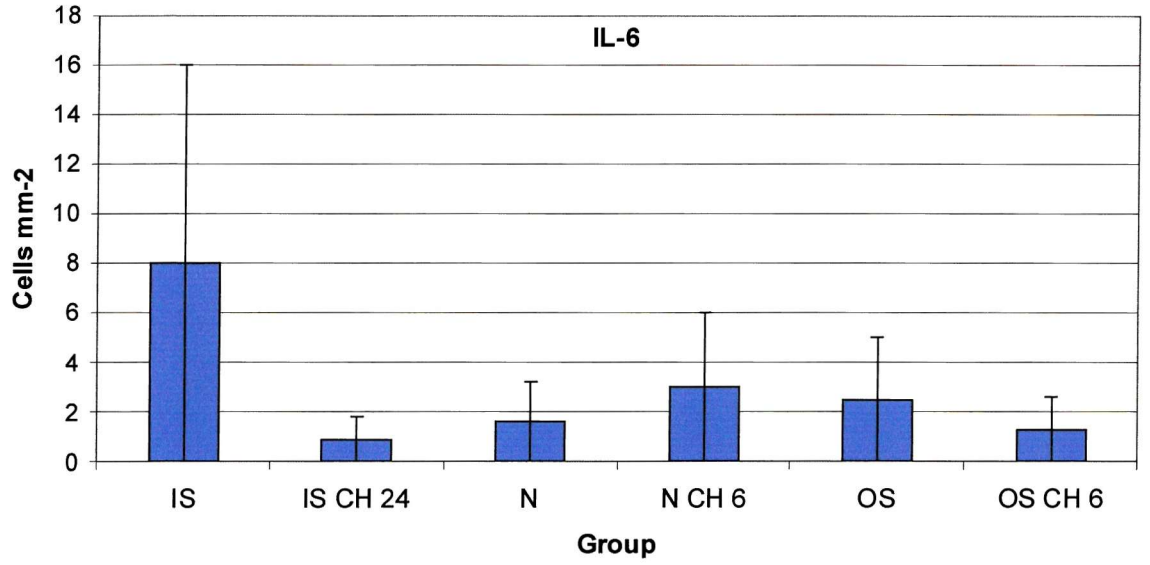
Legend

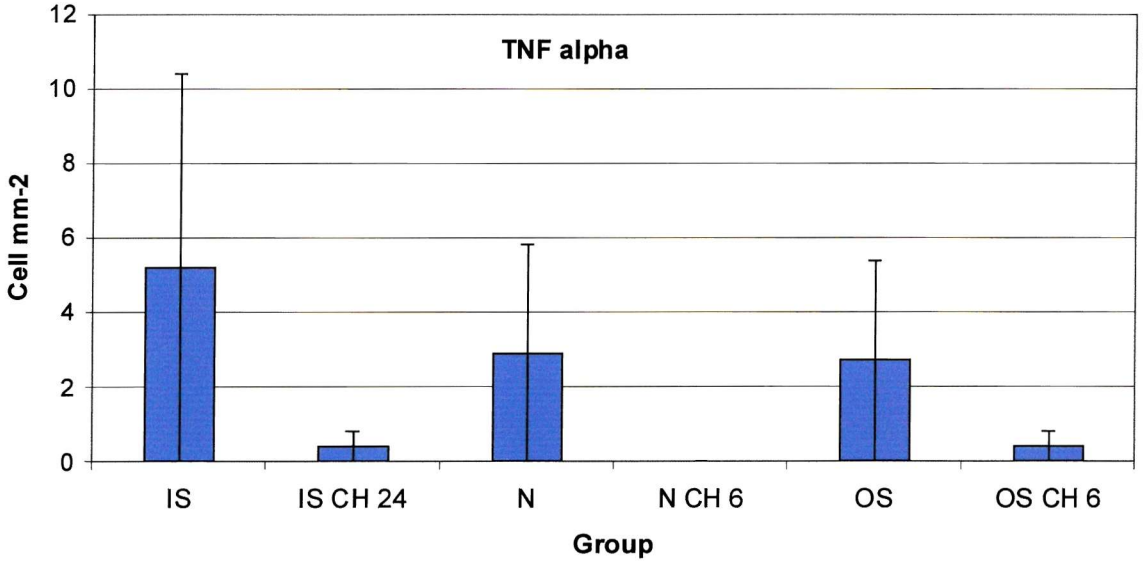
AA1, tryptase$^+$ mast cells; CHY, chymase$^+$ mast cells; EG2, eosinophils; NE, neutrophils; IS CH 24, symptomatic SAC subjects challenged and biopsied after 24 hours; N CH 6, normal subjects challenged and biopsied after 6 hours; OS CH 6, asymptomatic subjects challenged and biopsied after 6 hours; IS, symptomatic SAC subjects in season; OS, asymptomatic SAC subjects out of season; N, normal controls. Error bars show SEM for each count.

FIGURE 9.4

Mean cytokine counts for allergen challenged subjects, symptomatic and asymptomatic subjects with SAC, and controls







Legend

3H4, IL-4^+ cells using mAb 3H4; 4D9, IL-4^+ cells using mAb 4D9, IL-5, IL-5^+ cells; IL-6, IL-6^+ cells, TNFalpha, TNFα^+ cells; IS CH 24, symptomatic SAC subjects challenged and biopsied after 24 hours; N CH 6, normal subjects challenged and biopsied after 6 hours; OS CH 6, asymptomatic subjects challenged and biopsied after 6 hours; IS, symptomatic SAC subjects in season; OS, asymptomatic SAC subjects out of season; N, normal controls. Error bars show SEM for each count.

⌐*¬ $P < 0.05$

were challenged and biopsied at 6 hours or asymptomatic or symptomatic patients with SAC or normal controls. The mean number of AA1 positive mast cells was lower in out of season subjects challenged and biopsied at six hours (12.4 ± 1.0 mm^{-2}) than control subjects (19.9 ± 2.6 mm^{-2}), asymptomatic subjects out of season (22.9 ± 3.5 mm^{-2}), symptomatic in season subjects (27.3 ± 4.1 mm^{-2}) or symptomatic in season subjects challenged and biopsied at twenty four hours (22.2 ± 4.0 mm^{-2}) although these differences were not statistically significant.

The number of IL-4 positive cells labeled with the mAb 3H4 was significantly higher in symptomatic SAC patients (19.3 ± 2.9 mm^{-2}) than in asymptomatic SAC patients challenged and biopsied at 6 hours (6.9 ± 1.0 mm^{-2})($p = 0.0026$); in asymptomatic SAC patients challenged and biopsied at 24 hours (21.9 ± 4.6 mm^{-2}) compared with asymptomatic SAC patients challenged and biopsied at 6 hours (6.9 ± 1.0 mm^{-2})($p = 0.0022$) and in asymptomatic SAC patients (17.4 ± 2.2 mm^{-2}) compared with asymptomatic SAC patients challenged and biopsied at 6 hours (6.9 ± 1.0 mm^{-2})($p = 0.0003$). There was no statistical difference in the numbers of IL-4 positive cells labeled by the mAb 3H4 between symptomatic or asymptomatic patients with SAC and asymptomatic SAC patients challenged and biopsied at 24 hours.

The numbers of IL-5, IL-6 and TNFα positive cells did not differ significantly between groups.

9.4 Discussion

This study demonstrated that the symptoms of SAC could be clinically reproduced by CAC in a safe and consistent manner and that all SAC subjects demonstrated a statistically significant increase in symptom score 20 minutes following CAC. By aiming to elicit a symptom score ≥ 4, the highest probability of achieving a LPR was obtained. Although one subject experienced an adverse reaction to the challenge, the CAC protocol was modified to minimize the systemic effects of AC and no further adverse effects occurred. The study also enabled a visual scale of conjunctival hyperaemia and chemosis to be constructed to provide an objective grading of clinical conjunctival changes due to CAC. A late phase response was clinically evident in the out of season SAC patients challenged

and biopsied at six hours with no significant difference between their mean symptom scores at this point and at 20 minutes. This finding was in keeping with the findings of a clinical conjunctival LPR at six hours with high dose allergen challenge in experimental animals [Allansmith et al. 1984; Bonini et al.1987] and man [Bonini et al.1990; Bonini et al. 1994]. In these studies, maximal dose CAC produced symptoms of SAC at six hours which correlated with the number of eosinophils in the tear fluid in two out of the eleven atopic patients challenged.

Higher symptom scores were not evident for any other group after 20 minutes. Tissue specific changes in mast cell, eosinophil or neutrophil numbers however, were not observed at six hours, in fact mast cell numbers using both mAb's AA1 to tryptase and MAB1254 to chymase were lower in this group than any other. Since the nature of the study precluded more than one biopsy per eye being taken, it was possible that the optimal time point to study the LPR may have been missed.

The number of IL-4 positive cells labeled by the mAb 3H4 was significantly higher in symptomatic in season, asymptomatic out of season, and challenge patients with SAC biopsied 24 hours later, than in out of season patients challenged and biopsied at six hours. The mAb 3H4 labels IL-4 at the cell surface suggestive of IL- 4 export and the number of 3H4 positive mast cells has previously been shown to be significantly higher in symptomatic SAC patients compared with normals (Chapter 6). The relatively low number of 3H4 positive cells in the six hour challenge group therefore, provided no evidence that IL-4 release contributed to the clinical LPR observed. The lack of response may have been due to several factors:

- Flawed methodology in the CAC technique may have resulted in an insufficient allergen challenge. All patients however, responded significantly to CAC and for the OOS CH 6 group a clinical LPR was observed at six hours and both mast cell and 3H4 cell numbers in the in season patients challenged and biopsied at 24 hours were similar to symptomatic in season patients. Furthermore, in SAC symptoms are not episodic in response to distinct periods of high dose allergen exposure, but in keeping with a LPR described in bronchial [Dolovich et al.1973; Sedgwick et al. 1991]and nasal [Iliopoulos et al. 1990] mucosa and skin [Gleich 1982].

- Inappropriate choice of biopsy time. The six hour time point was based upon data from nasal allergen challenge demonstrating a LPR between 4 and 10 hours post challenge in 47% of subjects [Iliopoulos et al.1990], cutaneous allergen challenge in which an increase in inflammation was noted between six and 12 hours [Gleich 1982], bronchial biopsy evidence of increased numbers of mast cells, eosinophils, neutrophils but not $CD4^+$ cells six hours post challenge [Montefort et al.1994] and increases in conjunctival neutrophils six hours post challenge in experimental animals [Allansmith et al.1984] and increased numbers of eosinophils in tear fluid in humans six hours post challenge [Bonini et al.1990]. This study was the first to examine tissue changes in mast cell, eosinophil, neutrophil and cytokine positive cell numbers in human conjunctival biopsies and the possibility of significant tissue changes prior to or after six hours remained. Although not significant, mast cell numbers were highest in those symptomatic SAC patients challenged and biopsied at 24 hours, and in a cytological imprint study during natural allergen exposure, mast cell numbers were not noted to rise until after four to five days of pollen exposure [Pipkorn et al.1988].

- Lack of statistical power. The numbers of biopsies examined and cells counted may have been insufficient to demonstrate a difference in cell or cytokine numbers.

In summary, CAC reliably reproduced the clinical effects of SAC 20 minutes after challenge and generated a clinical LPR in asymptomatic out of season patients challenged and biopsied at six hours. No tissue evidence of changes in mast cell, eosinophil or neutrophil numbers supported this response however, and no differences in the numbers of IL-4, IL-5, IL-6 or TNFα positive cells were observed for any of the study groups.

CHAPTER 10

Discussion

The conjunctiva is the largest component of the ocular surface by area and by contrast with other ocular structures contains a high density of mast cells [Allansmith et al.1978]. The deposition of allergen on the conjunctival epithelium in susceptible individuals leads to the bridging of allergen-specific IgE bound to FcεRI receptors at the mast cell surface and subsequent mast cell degranulation and mediator release. This cascade of events is responsible for the itch, lacrimation, chemosis and hyperaemia of the conjunctiva which comprise the clinical entity of SAC. This condition is prevalent, unpleasant but not sight threatening and the mediators of the acute hypersensitivity response, histamine, PGD_2 and LTC_4 are well recognized as its central effectors. Mast cell research has demonstrated that this cell is a source of multifunctional cytokines. Evidence presented in Chapter 5 showed that as well as mast cell degranulation, conjunctival mast cell, as well as T cell numbers, were significantly raised in SAC and that the cardinal symptoms of SAC were related to mast cell numbers. Chapter 6 presented work which demonstrated that conjunctival mast cells are an important source of cytokine immunoreactivity. The majority of IL-4, IL-13, IL-5, IL-6 and TNFα positive cells in bulbar conjunctival biopsies were mast cells and importantly, IL-4 mRNA was also co-localized to mast cells, suggesting that both preformed and newly synthesized IL-4 may be produced. IL-4 has unique and key roles in regulating IgE antibody production and the development of effector T cell responses. The presence of IL-4 is required for T_H2-like cell growth and development and the class switching of B cells to IgE production, the IL-4 gene is closely related to that controlling IgE regulation, so the finding that more than 90% of immunoreactive IL-4 in conjunctival biopsies was localized to mast cells provides a mechanism for mast cell driven, local, IgE dysregulation to play a central role in the pathogenesis of SAC. Through the production and release of newly generated and preformed cytokines, mast cell degranulation would also have the capacity for the sustained upregulation of CAM's. The proinflammatory effects of mast cell products on endothelial tissue might thus extend beyond those due to the release of vasoactive amines. IL-4 is known to selectively upregulate P-selectin [Yao et al.1996] within minutes of release and VCAM-1 [Bacon et al.1998; Schleimer et al.

1992] over several hours, whilst TNFα is known to upregulate both ICAM-1 and VCAM-1 [Walsh et al.1991; Zhang et al. 1992]. This process would be expected to result in increased accumulation of lymphocytes to areas of allergic inflammation, consistent with the findings of Chapter 5. These findings then, may identify new mechanisms by which conjunctival mast cells could regulate LPR's which are thought to provide the link between type I hypersensitivity and the persistent symptoms experienced by patients with SAC. This link was explored in Chapter 9 where a clinical LPR was reliably reproduced by CAC using symptom score to titrate the dose of allergen.

The capacity for conjunctival mast cell activation to drive the immediate allergic response in SAC, to recruit T cells and other proinflammatory cells through the release of cytokines and CAM upregulation, and to stimulate a clinical LPR in the absence of the activation of other inflammatory cells places this cell at the center of the pathogenesis of SAC. Little however, is understood about the regulation of mast cell numbers or phenotype in man. To address these questions, the phenotype of mast cells in patients with SAC and normal controls, and the functional difference between mast cell phenotype with respect to cytokine immunoreactivity was investigated. The work presented in Chapter 7 showed that the majority of mast cells in conjunctiva from patients with SAC and normal controls were MC_{TC} and proportionally, the majority of IL-4 and IL-13 were co-localized to this phenotype. This work was supportive of evidence in human airway mucosa [Bradding et al.1995a] suggesting that IL-4 in bronchial and nasal mucosal mast cells co-localized to the MC_{TC} phenotype whilst IL-5 and IL-6 preferentially co-localized to MC_T. IL-13, like IL-4, has been shown to play important roles in the recruitment of inflammatory cells to sites of allergic inflammation through VCAM-1-dependent mechanisms [Ying et al. 1997] further supporting the hypothesis that mast cell subsets in man may be separated functionally by their cytokine profile rather than by ultrastructural changes alone.

It is not known whether the control of mast cell phenotype is determined in the bone marrow, lies at tissue level, or whether it can change following degranulation. As the circulating precursors of mast cells have not been identified, work in Chapter 8 focused on the tissue regulation of mast cells. The most important cytokine regulator of mast cell growth, development and function is known to be SCF [Galli 1990; Galli et al. 1993; Galli et al.1994a] and most of the reported literature regarding SCF has been obtained through

mast cell research. The key findings of Chapter 8 are that mast cells themselves are a source of this cytokine. This knowledge provides the first evidence in man, that SCF, the most important regulator of mast cell biology may be produced and released in an autocrine manner.

10.1 Cellular changes in SAC

Mast cells are ubiquitous tissue dwelling granulocytes which are capable of releasing the pre-formed and newly generated mediators of type I hypersensitivity reactions when activated. Mast cell degranulation in the absence of increased numbers of cells has been observed in the eyelids of experimental animals challenged with allergen [Allansmith et al.1984] and in bronchial and nasal mucosa [Djukanovic et al.1990] [Gomez et al.1986] from patients with allergic airways disease in man. Increased numbers of mast cells, eosinophils and T cells have been demonstrated in chronic allergic conjunctivitis [Allansmith et al.1979a; Maggi et al.1991; Morgan et al.1991] but information regarding conjunctival tissue changes in subjects with SAC however, is limited [Allansmith et al.1978]. In Chapter Five, the results of immunohistochemical analysis of bulbar conjunctival biopsies from normal and atopic patients with SAC in and out of the pollen season, are reported. Differences in symptom score between symptomatic patients and asymptomatic SAC patients and normal controls were highly significant ($p < 0.001$) and a trend toward increased mast cell numbers with increasing symptom score in symptomatic SAC patients was observed (Figure 5.1). High quality conjunctival histology (Figure 5.3) enabled sensitive and specific counts of mast cells, eosinophils, neutrophils and T cells to be made. These counts demonstrated a significant increase in mast cell and T cell numbers over normal subjects and higher numbers of mast cells in conjunctiva from symptomatic compared with asymptomatic subjects. Although a significant rise in T cell numbers was noted in both the conjunctival epithelium and substantia propria, T cells are known to be circulating immune cells whose numbers might be expected to rise during an allergic immune response. Evidence suggesting that this might be the case included immunohistochemical studies showing T cell infiltrates in chronic allergic conjunctivitis [Maggi et al.1991; Metz et al. 1996]. The finding that mast cell numbers were increased in conjunctiva from symptomatic subjects with SAC, despite the reported increase in mast cell degranulation in allergic conjunctivitis, provided strong evidence to support the

hypothesis that this cell contributed to the pathophysiology of SAC. This finding is particularly relevant given that the conjunctiva is known to contain a high proportion of inflammatory cells [Allansmith et al.1978] and that mast cell degranulation in the absence of increased cell numbers has been reported in many conditions in which T cell and eosinophil numbers have been shown to be increased.

10.2 Mast cell cytokines

Cytokines are immunomodulatory molecules, which are produced by, and act upon, all cells of the immune system. The complexity of the actions of cytokines is in part due to their functions being modified by the presence of other cytokines, thus these locally acting molecules are often described as forming 'networks' [Balkwill et al.1989]. Cytokine production by T cells is well described and the secretion of cytokines adds to the capability of this class of immune cell to regulate immune reactions. More recently however, mast cells have been shown to produce, store and release cytokines, first in cell lines and tissue culture [Chung et al.1986; Plaut et al.1989], then experimental animals [Gordon et al.1990a] and later in man [Bradding et al.1992; Bradding et al.1993; Bradding et al.1995a; Burd et al.1995; Gordon et al.1990a; Moller et al.1993]. This evidence and work demonstrating that T_H2 cytokines and IL-4 in particular, play central roles in upregulating allergic responses [Finkelman et al. 1988; Jeppson et al.1998] was suggestive that mast cells could potentially exert a powerful influence on the conjunctival allergic response by producing T_H2-like cytokines. Furthermore, as the gene regulating IgE production is closely linked with *IL4* [Marsh et al.1994] and many forms of allergic conjunctivitis are characterized by IgE dysregulation, the source of rapidly available, locally acting IL-4 is a key issue in understanding the pathogenesis of allergic conjunctivitis. By adapting the techniques of IHC and ISH, the specific demands of working with conjunctival biopsies could be met to produce histology of high enough quality to enable thin serial sections of tissue to be examined. Chapter 6 describes the cytokine profile of human conjunctiva in normal and atopic patients and the results of co-localization studies of cytokines and mast cells known to be important in allergic disease. The total number of IL-4 and IL-6 positive cells were significantly elevated in conjunctiva from symptomatic SAC subjects compared with normals ($p = 0.0013$ and $p = 0.015$ respectively) and IL-4, IL-5, IL-6, IL-8, TNFα and IL-13 were co-localized to

conjunctival mast cells (Figures 6.2, 6.3, 6.4). In the case of IL-4, IL-5, IL-6 and IL-13 the majority of cytokine positive cells in all groups were mast cells suggesting that these cells might be an important source of cytokines which could be secreted immediately during cell activation. Wilson et al. [Wilson et al. 2000] demonstrated that IL-4 was stored within the matrix of human mast cell granules using immunogold electron microscopy and suggested that IL-4 might be replenished on regranulation. The *in-situ* hybridization and double IHC studies reported in Chapter 6 provided further supportive evidence that conjunctival mast cells are not only an important source of IL-4 but capable of synthesizing this cytokine. Since T cells require a priming pulse of IL-4 to activate STAT-6 and ensure the commitment of the T_H2 lineage [Swain et al.1990] via GATA3 [Rao et al.2000] dependant mechanisms, the release of preformed IL-4 into the conjunctival microenvironment provides a powerful potential mechanism to contribute to IgE upregulation and a T_H2-type immune response prior to the recruitment and activation of T cells. Through the selective upregulation of VCAM-1 via IL-4 and TNFα, mast cell cytokine release might contribute to the recruitment of other circulating granulocytes although neither eosinophil nor neutrophil numbers were elevated in symptomatic patients with SAC over asymptomatic or normal control subjects (Chapter 5).

These findings contribute to our understanding of the pathophysiology of SAC but also have implications for the direction of therapeutic intervention studies aimed at modifying the disease process of SAC through cytokine and mediator targeting. Based upon the techniques and tissue findings of the studies reported herein, work investigating the effects of the SAC disease modifying agents nedocromil sodium and levocabastine has been initiated [Ahluwalia et al. 2001] utilizing the objective measures of disease activity in addition to subjective analysis of symptom scores.

10.3 Mast cell heterogeneity

Having established that conjunctival mast cells are an important source of T_H2-like cytokines (Chapter 6), the aim of the work in Chapter 7 was to investigate whether the pattern of cytokine expression differed between mast cell sub-types. Mast cell heterogeneity has been described in experimental animals, at different tissue locations within species and between mast cell sub-types at the same location in man [Irani et al.1989; Irani et al.1986]. The division of human mast cells into MC_T and MC_{TC} by neutral protease content has been the standard way of sub-classifying mast cells in man [Irani et al.1989; Irani et al.1986; Irani et al.1990b]. As knowledge regarding mast cell protease function is limited however, and mast cells have been identified in culture [Li et al.1996b] which do not fall into either category, the evidence presented in Chapter 7, is important because it is suggestive of differences of functionality between classes of mast cell rather than histological appearance alone.

Human conjunctival mast cells have been reported to show differences in the proportion of MC_T and MC_{TC} sub-types in seasonal and chronic allergic conjunctivitis [Baddeley et al.1995; Irani et al.1990b] compared with normals. In contrast to Baddely's earlier work [Baddeley et al.1995], the work described in Chapter 7 found that the majority of mast cells in normal, symptomatic and asymptomatic patients with SAC were MC_{TC} cells, although as previously [Baddeley et al.1995], the highest proportion of MC_T cells were observed in symptomatic SAC patients. Reasons for this difference might have included differences in the method of co-localizing the tryptase and chymase granules. By using the cytokine co-localization methodology described in Chapter 4, over 90% of cells could be identified in adjacent sequential sections, the highest proportion reported to date [Anderson et al. 2001].

The results presented in Chapter 7 support Bradding's observations in bronchial and nasal mucosal biopsies, that IL-4 was preferentially and significantly localized to the MC_{TC} phenotype [Bradding et al.1995a] using mAb's to both cytoplasmic and cell surface IL-4, whilst IL-5 and IL-6 tended to co-localize to the MC_T subset (although these changes did not achieve statistical significance in conjunctival biopsies). Like IL-4^+ cells, the majority of IL-13^+ cells were MC_{TC} in phenotype although this was not significantly significant.

As IL-4 and IL-13 share almost identical actions and close receptor homology [de Vries et al.1995], these findings were consistent with the hypothesis of functional heterogeneity and in the case of IL-13, previously undescribed in man. Since circulating mast cell precursors have not been identified to date, the mechanism for the observed differences in the proportion of mast cell sub-populations remains unknown. In particular, it is not known whether these observations reflect the results of selective recruitment of cells destined to differentiate into one particular phenotype, or tissue directed changes in phenotype of mature or regranulating cells. As the pattern of mast cell regranulation into selective phenotypes is also only partly understood, further work to elucidate the patterns of mast cell regranulation in normal human conjunctiva with time are required. Human mast cells expressing chymase only have been cultured using recombinant SCF suggesting that differential expression of proteases may be possible *in-vivo*.

10.4 Mast cell regulation

Stem cell factor plays widely diverse biological roles including the development and function of the central nervous system, melanocyte development, haematopoiesis and gametogenesis [Galli et al.1993]. The most complete picture regarding the interactions of SCF and its ligand, *c-kit*, have however, been gained in mast cell research. Stem cell factor is the major cytokine regulator of mast cell growth, maturation, survival and function [Bischoff et al.1992; Columbo et al.1992; Gagari et al.1997; Iemura et al.1994; Irani et al.1992; Meininger et al.1992; Mitsui et al.1993; Okayama et al.1995; Valent et al.1992] and the work of Kitamura and others has demonstrated that certain key biological responses e.g. IgE-mediated inflammation, parasite immunity and bacterial clearance depend upon, or are severely attenuated in the absence of, either SCF or its ligand [Kitamura et al.1978; Kitamura et al.1979; Kitamura et al. 1995; Malaviya et al.1996; Wershil et al. 1985; Wershil et al. 1994]. The heterogeneous nature of mast cells and the absence of data in man has, however, resulted in much of our knowledge of mast cell : SCF interactions being derived from studies on experimental animals or from the actions of fibroblast-derived or recombinant SCF on mast cell cultures. To better understand the biology of this cytokine in man, the aim of the work in Chapter 9 was to determine whether mast cells were capable of producing and storing SCF. The central finding of this work, that mast cells of both sub-types from skin and conjunctiva expressed SCF, adds

significantly to our knowledge of the biological potential of this cell. Of particular relevance was that as neither sensitization nor cross-linkage of FcεR1 receptor on lung MC's has been shown to alter SCF mRNA expression or SCF secretion, the regulation of SCF expression in human mast cells may be independent of high affinity IgE -mediated pathways [Zhang et al. 1998]. As SCF enhances IgE-dependent human mast cell mediator release [Bischoff et al.1992; Columbo et al.1992] the interactions between SCF expression and IgE clearly merit further investigation. The findings of Chapter 8 add new knowledge to the understanding of mast cell autoregulation. Since mast cells are involved in a wide variety of pathological processes including acute inflammation and repair, these findings not only broaden the possibilities for further work studying the biology of *c-kit*: *c-kit* ligand interactions using the mast cell as a model, but also the direct investigation of clinical disease processes of mast cell accumulation e.g. systemic mastocytosis [Austen 1992] in the light of the possibility of autocrine dysregulation of SCF production.

10.5 Conjunctival allergen challenge

Conjunctival allergen challenge represents a human model for studying allergic diseases including allergic conjunctivitis [Abelson et al.1990; Bonini et al.1994; Friedlaender et al. 1996], a powerful tool for evaluating anti-allergic drugs [Ahluwalia et al.2001], a method for collecting inflammatory mediators and cells, and a method of establishing the diagnosis of SAC. Conjunctival allergen challenge is highly reproducible when symptom scores are evaluated, but like SAC, there is very little objective evidence of associated cellular or tissue changes to date. The aims of Chapter 10 were parallel to Chapter 1; to seek evidence of tissue cell and cytokine changes associated with CAC and if possible, to relate them to the symptoms of SAC and the observations described in Chapter 5 and 6. It was recognized that since tissue would be studied, only one sample could be taken from each subject and that his would limit the data analysis to a single time point. The analysis of multiple samples from the same subject at various times after CAC would have yielded longitudinal data and would also have provided paired data to strengthen the statistical analysis. Single tissue samples were chosen however, because tissue changes had not been previously investigated following CAC and the results would also be comparable to those obtained from the work performed in Chapters 5,6,7 and 8. Furthermore, there are well described methodological problems with tear and epithelial cell collection and

analysis [Aichane et al.1993; Pipkorn et al.1988; Tuft et al.1989b], in particular, it was recognized that CAC and mediator release of histamine and PGD_2, were poorly correlated [Aichane et al.1993; Proud et al.1990].

The principal findings of Chapter 9 were that CAC reliably and consistently produced a clinical LPR evidenced by symptom score, but failed to demonstrate associated tissue changes. These findings failed, therefore, to support the hypothesis that the conjunctival LPR provides a link between conjunctival type I hypersensitivity and the continuous allergic inflammation of the conjunctiva in SAC evidenced by the cell and cytokine profile described earlier. This may have been due to lack of power of the study to detect statistically significant changes in cell numbers, lack of a true response, poor choice of sampling time or incorrect method of assessing the changes. As changes in cell numbers in previous chapters had achieve statistical significance, patients experienced symptoms of a LPR which were highly significant compared with controls and baseline measurement and most authors reporting a LPR in skin, airway and conjunctiva had monitored a response peaking between six and eight hours and lasting up to 48 hours, the former hypotheses seemed less likely. More probably, as the conjunctival LPR is highly variable between individuals [Bonini et al.1989; Bonini et al.1990; Bonini et al.1994; Proud et al.1990] the numbers of patients studied was too small to detect significant changes in cell numbers if those changes were present. Interestingly, work examining the expression of ICAM-1 (CD54) on conjunctival epithelial cells [Bacon et al.1998; Ciprandi et al.1993b; Ciprandi et al.1995a] has shown increased expression during natural allergen exposure and allergen challenge and also a positive correlation between the levels of ICAM-1 expression and the degree of lymphocyte and granulocyte infiltrate. A powerful stimulus for ICAM-1 expression is TNFα and this cytokine was co-localized to conjunctival mast cells (Chapter 6) providing another possible mechanism for mast cell degranulation to selectively recruit inflammatory cells to areas of allergic inflammation.

The work in this thesis provides novel evidence in human conjunctival tissue that mast cells not only release the mediators of type I hypersensitivity but are a significant source of cytokines which may selectively bias this tissue toward a T_H2-type immune response. Furthermore, the findings of this work suggest that conjunctival mast cells may be functionally heterogeneous, evidenced by differences in their pattern of cytokine

expression. By demonstrating that human mast cells are a source of their most powerful regulatory cytokine, SCF, new insight into mast cell biology has been gained, in particular, that regulation of mast cell function may be under autocrine influence. Although CAC provides a standard human model to investigate the clinical symptoms of SAC, no evidence of significant tissue changes in subjects undergoing CAC was observed. The conjunctival LPR was reproduced consistently however, enabling a photographic scale of response to be constructed and preliminary therapeutic intervention studies utilizing this methodology to commence.

This thesis presents novel findings regarding the role of the mast cell in SAC. The known capacity of mast cells to produce mediators capable of initiating Type I hypersensitivity responses has been extended to include the capacity to release cytokines in an immediate and sustained manner capable of the recruitment of inflammatory cells to the conjunctiva and drive the LPR in the absence of the activation of other inflammatory cells. Further evidence has been presented to show that conjunctival mast cells display functional heterogeneity with regard to their pattern of cytokine immunoreactivity in addition to their known ultrastructural differences, and that human mast cells are a source of SCF, the most important regulator of mast cell biology.

APPENDIX 1

Solutions

Solutions used during IHC performed on GMA embedded tissue

Poly-L-lysine slide coating

10 mls	0.1% Poly-L-lysine (Sigma, Poole, UK)
90 mls	Reverse osmosis water (ROW)

Slides were placed in steel racks and immersed in the above solution for 5 minutes. They were then allowed to air dry under cover overnight and re-packed into boxes. Slides were not used after 6 months.

Acetone and protease inhibitors

500 mls	Acetone (Merck)
175 mgs	2mM Phenyl methyl sulphonyl fluoride (Sigma)
1.85 mgs	20mM Iodoacetamide (Sigma)

These were stored in glass at -20^{o}C.

Anhydrous acetone

Acetone

Molecular seive type 4 (crystalline sodium alumino-silicate) self indicating 1/16" pellets (BDH Ltd., Poole, UK)

Stock acetone passed over a molecular seive (type 4) at room temperature.

GMA infiltrating and embedding solutions

Solutions supplied as a kit from Park Sciences Ltd., Northampton, UK.

Infiltration solution

5%	Methyl benzoate (Merck)
10mls	JB4 Solution A, GMA monomer

Embedding solution

10mls	JB4 Solution A, GMA monomer
250µls	JB4 Solution B, N,N-dimethylaniline in PEG 400
45mg	benzoyl peroxide

Solutions were prepared immediately prior to use.

Endogenous peroxidase solution

10mls	0.1% Sodium azide (NaN_3) 0.05g NaN_3 (BDH) + 500mls ROW
100µls	30% Hydrogen peroxide (H_2O_2) in ROW

Culture medium

30mls	10% Fetal Calf Serum γ globulin free (GIBCO, Paisley, Scotland)
270mls	Dulbecco's Modified Eagles Medium with L-glutamine
1.0g	Glucose/litre without sodium bicarbonate (Sigma)
3.5g	1% Bovine serum albumin (BSA) (Sigma)

The BSA was stirred into the solution. Aliquotes were then stored at -20°C and thawed immediately prior to use.

TRIS/HCL Buffered saline (TBS) pH7.6

80g	Sodium chloride
6.05g	TRIS hydroxymethylmethylamine (TRIS)
38ml	1N Hydrochloric acid (HCL)
10L	Distilled water

Salts dissolved in 1L of distilled water and pH adjusted to 7.75 with HCL, solution then added to remaining 9L. Final pH checked at pH7.6.

All chemicals supplied by BDH Ltd, Poole, UK.

Streptavidin biotin - enzyme complexes (SABC)

Tris HCL solution

13ml	0.2M Tris (BDH)
13ml	0.1N HCL (BDH)
19ml	ROW

Tris stored at 4°C. Streptavidin and biotin diluted in Tris HCL at previously determined optimal dilution. Solution allowed to complex for at least 30 mins prior to use.

Aminoethylcarbazole (AEC) / hydrogen peroxide (H_2O_2) solution

1ml	0.4% AEC	0.05g AEC powder (Sigma) in 12.5ml dimethylformamide (DMF)(Merck)
15µl	30% H_2O_2 (BDH)	
14ml	0.1M Acetate buffer	

This was prepared and filtered immediately prior to use.

Fast Red Chromogen

10ml	Veronal acetate buffer
5mg	Fast red TR salt (Sigma)
2.3mg	Levamisole (Sigma)
5mg	Napthol AS Bi-phosphate (Sigma)
15µl	DMF (Merck)

Napthol AS Bi-phosphate made up in DMF immediately prior to use and added to other compounds. Solution filtered prior to application.

Acetate buffer pH5.2

40ml	0.1M sodium acetate (BDH)	
10ml	0.1M acetic acid	10ml acetic acid (BDH) in 90ml ROW

Veronal Acetate Buffer

8.1g/L 30mM sodium acetate trihydrate

6.1g/L 30mM sodium barbitone

5.8g/L 100mM sodium chloride

10.1g/L 50mM magnesium chloride hexahydrate

All chemicals from Merck

pH adjusted to 9.2, buffer stored at 4°C. Filter before use.

Slide mounting solution for dual IHC

1 tabletCitiflour in 300ml TBS

10mls Mowiol

Solutions used during ISH performed on paraffin embedded tissue

All solutions of at least Analar quality. All solutions prepared using DEPC treated water. Reagents supplied by Merck unless stated to the contrary.

Fixative

10% neutral buffered formalin

Wax

Speciwax Histological paraffin wax, melting point 56°C (Speci-microsystems, Surrey, UK.).

0.1% Diethylpyrocarbonate (DEPC) water

1mL DEPC

10L ROW

Mixed and left overnight at r.t. Autoclaved and then stored at r.t.

10X PE (modified)

This does not contain sodium dodecyl sulphate or BSA.

g / 500ml DEPC-water	Concentration	
	0.5M	Tris-HCl buffer pH 7.5
5g	1%	Sodium pyrophosphate
10g	2%	Polyvinylpyrolidone
10g	2%	Ficoll (mwt 400,000)
9.306g	50mM	Etheylenediaminetetra-acid disodium salt (EDTA)

Dissolved at 65°C and held at this temperature for 15mins. Stored at r.t.

10X Phosphate buffered saline (PBS)

g / L DEPC-water	Concentration	
76g	1.3M	Sodium chloride
11g	70mM	Sodium dihydrogen orthophosphate
5.3g	30mM	Di-sodium hydrogen orthophosphate

Adjusted to pH 7.6. Stored at r.t.

AP buffer

2ml	1M Tris/HCL pH9	(10X dilution)
1ml	1M $MgCl_2$	(20X dilution)
0.4ml	5M NaCl	(50X dilution)
16.6ml	ROW	

To make 20ml buffer. Prepared immediately prior to use.

20X Standard saline citrate (SSC)

g / 2L DEPC-water	Concentration	
350.64g	3M	Sodium chloride
176.46g	0.3M	Tri-sodium citrate

Adjusted to pH 7 with sodium hydroxide

Sheared salmon sperm DNA

100mg salmon sperm (Sigma) / 10ml ultra-pure water.

Dissolved by stirring overnight at 4°C on a roller mixture.

DNA sheared by sonification using an MSE 150 miniprep apparatus used at thirty second cycles with a cooling stage in between each cycle. This was achieved by placing the sample in an ice-bath. Boiled for 10 mins and cooled on ice immediately prior to use.

10X Tris EDTA (TE)

g / 500ml	Concentration
DEPC-water	
6.057g	0.1M Tris
1.86g	0.01M EDTA

Tris dissolved first then EDTA added and solution adjusted to pH 8.

Tris acetate buffer (TAE Buffer)

Stock solution X50

242g	Tris-base
57.1ml	Glacial acetic acid
100ml	0.5M EDTA

Solution adjusted to pH8.

Working solution (X1)

0.04M	Tris-acetate
0.001M	EDTA

Bromophenol blue (1 in 10 for each well) (X10)

50%	Glycerol in TE
0.25%	Bromophenol blue

Solution is blue when pH=7

Agarose gel 1.2% in TAE buffer

0.2pg	Agarose
20ml	H2O
400µl	50X TAE

Ethidium bromide solution

1mg	Ethidium bromide in 1 ml TAE buffer

Running buffer for agarose gel

5ml	(50X) TAE
125µl	Ethidium bromide solution
245ml	H2O

Hybridization buffer (1ml) used for IL4 riboprobe

250µl	20X SSC
100µl	50X Denhardt's solution
100µl	2.5mg/ml tRNA (Yeast transfer RNA)
8µl	0.5M EDTA
500µl	formamide
25µl	10mg/ml salmon sperm DNA (sheared and denatured, see above)
17µl	DEPC.d H_2O

Hybridization buffer (1ml) used for SCF oligoprobe

500µl	50% formamide
	10% dextran sulphate
	0.6M NaCl
	50mM Tris HCl pH7.5
	0.1% Na_2HPO_4
	0.2% Polyvinylpyrrolidone and Ficoll
	5mM EDTA
25µl	150 µg/ml salmon sperm DNA (sheared and denatured, see above)

Reagents used for Conjunctival Allergen Challenge

Mixed grass pollen (Allerayde in association with HAL, formerly Soluprick SQ, ALK Laboratories, Denmark)

Composition:

Agrostis stofonisera	(bent grass)
Anthoxanthum odoratum	(sweet vernal)
Dactylis glomerata	(June / orchard grass, cox foot)
Lolium perennie	(rye grass)
Arrhenatherum elatius	(wild oat)
Festuca rubra	
Poa pratensis	
Secale cereale	
Holcus lanatus	(velvet grass)
Phleum pratense	(timothy grass)

Dermatophagoides pterontyssinus
cat dander
histamine (positive control)
saline and glycerol diluent (negative control)

Soluprick SQ, ALK Laboratories, Denmark

Aspergillus fumigatus
mixed tree pollen
dog dander

Miles Inc, Elkhart, IN, USA

APPENDIX 2

TABLE A1 Cell recount data

Masked Biopsy No:	True Biopsy Number	Average Masked Count	Average True Count	Masked Biopsy No:	True Biopsy Number	Average Masked Count	Average True Count
1	39	23.5	24.0	48	66	15.0	21.0
2	39	11.0	12.0	49	66	0.0	0.0
3	39	0.0	9.0	50	66	0.0	1.0
4	39	17.0	25.5	51	67	1.5	2.0
5	40	2.5	3.5	52	67	30.0	31.5
6	40	0.5	0.0	53	67	6.5	8.5
7	40	28.0	32.5	54	68	0.0	0.0
8	40	1.5	1.0	55	68	0.0	0.0
9	42	0.0	0.0	56	68	0.0	0.0
10	42	8.5	7.0	57	68	14.0	14.5
11	42	4.5	4.0	58	69	0.0	0.0
12	42	13.0	8.0	59	69	13.0	14.5
13	43	0.0	0.0	60	69	0.0	0.0
14	43	6.0	4.0	61	70	11.5	12.5
15	44	3.5	4.5	62	70	17.5	23.0
16	44	5.0	8.0	63	70	22.5	26.5
17	45	0.5	2.0	64	71	1.5	1.5
18	45	16.0	15.0	65	71	0.0	0.0
19	45	0.5	3.5	66	71	6.0	6.0
20	48	16.5	29.5	67	78	10.5	8.5
21	48	15.0	22.5	68	78	9.0	7.5
22	49	16.0	32.5	69	78	5.0	7.0
23	49	0.0	0.0	70	79	16.5	17.0
24	49	4.0	7.5	71	79	4.0	3.5

Masked Biopsy No:	True Biopsy Number	Average Masked Count	Average True Count	Masked Biopsy No:	True Biopsy Number	Average Masked Count	Average True Count
25	51	0.0	1.5	72	79	2.0	0.5
26	51	0.0	0.0	73	81	13.0	9.5
27	53	29.0	25.0	74	81	6.5	0.0
28	53	5.0	15.0	75	81	51.0	29.5
29	53	26.5	30.0	76	82	17.0	17.0
30	54	0.0	0.0	77	82	57.5	40.5
31	54	0.0	0.5	78	82	57.5	46.0
32	55	15.5	19.0	79	83	33.5	25.5
33	55	4.5	3.5	80	83	1.0	2.5
34	55	23.0	25.0	81	83	0.0	0.0
35	55	0.0	0.5	82	84	0.0	0.0
36	63	5.5	7.5	83	84	14.5	10.5
37	63	10.5	11.0	84	84	1.5	3.5
38	63	7.0	14.0	85	85	0.0	0.0
39	63	0.0	0.0	86	85	0.0	0.5
40	63	0.0	0.0	87	85	38.5	40.0
41	63	0.0	0.0	88	85	0.0	0.0
42	64	0.0	0.0	89	91	10.5	8.5
43	64	0.0	0.0	90	91	45.0	48.0
44	64	0.0	0.0	91	91	4.0	7.5
45	65	0.0	0.0	92	93	0.5	0.0
46	65	0.0	0.0	93	93	0.0	0.0
47	65	0.0	0.0				

APPENDIX 3

Gene and probe sequences

The nucleic acid sequence for human stem cell factor is given in Figure A1 (based on [Martin et al.1990].

Human stem cell factor oligoprobe sequences are given in Figure A2

Human interleukin-4 gene nucleic acid sequence is given in Figure A3 (based on [Yokata 1986])

Human interleukin-4 riboprobe sense sequence is given in Figure A4

FIGURE A1

The nucleic acid sequence for the human stem cell factor gene

ccgcctcgcg	ccgagactag	aagcgctgcg	ggaagcaggg	acagtggaga	gggcgctgcg	60
ctcgggctac	ccaatgcgtg	gactatctgc	cgccgctctt	cgtgcaatat	gctggagctc	120
cagaacagct	aaacggagtc	gccacaccac	tgtttgtgct	ggatcgcagc	gctgcctttc	180
cttatgaaga	agacacaaac	ttggattctc	acttgcattt	atcttcagct	gctcctattt	240
aatcctctag	tcaaaactga	agggatctgc	aggaatcgtg	tgactaataa	tataaaagac	300
atcactaaat	tggtggcaaa	tcttccaaaa	gactacatga	taaccctcaa	atatgtccca	360
gggatggatg	ttttgccaag	tcattgttgg	ataagcgaga	tggtagtaca	attgtcagac	420
agcttgactg	atcttctgga	caagttttca	aatatttctg	aaggcttgag	taattattcc	480
atcatagaca	aacttgtgaa	tatagtcgat	gaccttgtgg	agtgcgtcaa	agaaaactca	540
tctaaggatc	taaaaaaatc	attcaagagc	ccagaaccca	ggctctttac	tcctgaagaa	600
ttctttagaa	tttttaatag	atccattgat	gccttcaagg	actttgtagt	ggcatctgaa	660
actagtgatt	gtgtggtttc	ttcaacatta	agtcctgaga	aagattccat	agtcagtgtc	720
acaaaaccat	ttatgttacc	ccctgttgca	gccagctccc	ttaggaatga	cagcagtagc	780
agtaatagga	aggccaaaaa	tccccctgga	gactccagcc	tacactgggc	agccatggca	840
ttgccagcat	tgttttctct	tataattggc	tttgcttttg	gagccttata	ctggaagaag	900
agacagccaa	gtcttacaag	ggcagttgaa	aatatacaaa	ttaatgaaga	ggataatgag	960
ataagtatgt	tgcaagagaa	agagagagag	tttcaagaag	tgtaaattgt	ggcttgtatc	1020
aacactgtta	ctttcgtaca	ttggctggta	acagttcatg	tttgcttcat	aaatgaagca	1080
gctttaaaca	aattcatatt	ctgtctggag	tgacagacca	catctttatc	tgttcttgct	1140
acccatgact	ttatatggat	gattcagaaa	ttggaacaga	atgttttact	gtgaaactgg	1200
cactgaatta	atcatctata	aagaagaact	tgcatggagc	aggactctat	tttaaggact	1260
tgtttacttg	ggtctcattt	agaacttgca	gctgatgttg	gaagagaaag	cacgtgtctc	1320
agactgcatg	taccatttgc	atggctccag	aaatgtctaa	atgctgaaaa	aacacctagc	1380
tttattcttc	agatacaaac	tgcag				1405

Legend

Based on Martin FH, Suggs SV, Langley KE, Lu HS, Ting J, Okino KH, Morris CF, McNiece IK, Jacobsen FW, Mendiaz EA. Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell.* 1990; 63:203-211.

FIGURE A2

Human stem cell factor oligoprobe sequences

Probe 1 (P1) sense

gagcccgatg ggttacgcac ctgatagacg

P2 antisense (342-347 in mRNA sequence)

attgggagtt tatacagggt ccctacctac

P3 sense

taaccctcaa atatgtccca gggatggatg

P4 antisense (742-771 in mRNA sequence)

ggtcgaggg aatccttact gtcgtcatcg g

FIGURE A3

Human interleukin-4 gene nucleic acid sequence

5'

gatcgttagc ttctcctgat aaactaattg cctcacattg tcactgcaaa tcgacaccta tta
atg ggt ctc acc tcg caa ctg ctt ccc cct ctg ttc ttc ctg cta gca tgt gcc
ggc aac ttt gtc cac gga [cac aag tgc gat atc acc tta cag gag atc atc aaa
act ttg aac atc ctg aca gag cag aag act ctg tgc acc gag ttg acc gta aca
gac atg ttt gct gcc tgc aat aac aca act gag aag gaa acc ttc tgc agg gct
gcg act gtg ctc cgg gag ttc tag agc cac cat gag aag gac act cgc tgc ctg
ggt gcg act gca cag cag ttc cac agg cac aag cag ctg atc cga ttc ctg aaa
cgg ctc gac agg aac ctc tgg ggc ctg gcg ggc ttg aat tcc tgt cct gtg aag
gaa gcc aac gag agt acg ttg gaa aac ttc ttg gaa agg cta aag acg atc atg
aga gag aaa tat tca aag tgt tcg agc tga] atattttaat ttatgagttt ttgatagctt
tatttttaa gtatttatat atttataact catcataaaa taaagtatat atagaatcta aaa 3'

Legend

Based on Yokata T. Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell- and T-cell-stimulating activities. *Proc Natl Acad Sci USA.* 1986; 83:5894-5898.

FIGURE A4

Human interleukin-4 riboprobe sense sequence

aagctt acctgccatg-
cac aag tgc gat atc acc tta cag gag atc atc aaa
act ttg aac atc ctg aca gag cag aag act ctg tgc acc gag ttg acc gta aca
gac atg ttt gct gcc tgc aat aac aca act gag aag gaa acc ttc tgc agg gct
gcg act gtg ctc cgg gag ttc tag agc cac cat gag aag gac act cgc tgc ctg
ggt gcg act gca cag cag ttc cac agg cac aag cag ctg atc cga ttc ctg aaa
cgg ctc gac agg aac ctc tgg ggc ctg gcg ggc tt **g aat tc** c tgt cct gtg aag
gaa gcc aac gag agt acg ttg gaa aac ttc ttg gaa agg cta aag acg atc atg
aga gag aaa tat tca aag tgt tcg agc tga

Legend

The first line is not included in the IL4 gene sequence but was included in the probe as it was the sequence included by using the restriction enzyme Hind III which cleaved at the site highlighted in bold text (**aagctt**). The second sequence highlighted in bold text was the site cleaved by the restriction enzyme EcoR1.

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INDEX