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Local isotype switching to IgE within allergic nasal mucosa in response to allergen exposure.

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'In life the beginning and end are determined.
It is what occurs in the interim, who we meet and
what we do, that charts our course'.

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Abstract

Patients with allergic rhinitis exhibit an exaggerated nasal response to allergen, in large part a consequence of IgE-mediated inflammatory cell activation. IgE within the tissue is considered to derive from IgE-bound inflammatory and/or IgE+ plasma cells. Resting B lymphocytes and cells expressing IL-4 and IL-13, cytokines that induce B cells to undergo ε germline transcription and isotype switching to IgE, are present within allergic nasal mucosa. As such, it has been hypothesized here that *local* isotype switching to IgE may occur at this site. Probes to detect the ε germline transcript (Iε RNA), a pre-requisite for isotype switch recombination, the mature ε transcript (Cε RNA) and IL-4 and IL-13 mRNA were employed. Since steroids reduce the number of cells expressing these cytokines, the effect of pre-treating patients with fluticasone propionate (FP) was also investigated.

Nasal biopsies were obtained from seasonal allergic rhinitis patients before and following *in vivo* allergen challenge or natural exposure during the pollen season. To confirm local RNA synthesis, nasal tissue was challenged with allergen *ex vivo*. Immunocytochemistry (ICC) confirmed the presence of B cells within the nasal tissue. *In situ* hybidization (ISH) demonstrated increases in the number of Cε and IL-4 mRNA and the appearance of Iε RNA+ cells in nasal tissue from placebo-treated patients following *in vivo* allergen exposure, but not those pre-treated with FP. *Ex vivo* allergen challenge also resulted in higher numbers of Cε, Iε, IL-4 and IL-13 RNA+ cells in allergen-stimulated compared to unstimulated tissue. With simultaneous ICC/ISH, CD20+/Cε+ (37%) and CD20+/Iε+ (32%) were observed,

while the presence of mature ε mRNA (Cε+/Iε-) was confirmed using double ISH. Furthermore, IL-4 was associated primarily with T cells (≅70%) and mast cells (≅32%), while these cell types also produced IL-13 (≅44%, ≅18%).

It is demonstrated here that the ε germline transcript, mature ε mRNA and IL-4 and IL-13 mRNA are synthesized locally within allergic nasal mucosa and that steroids inhibit their production. These results indicate that events within the nasal mucosa itself play a primary role in regulating the allergic response at this site and emphasize the importance of focusing on this *local* regulation when designing future diagnostic and therapeutic strategies.

RESUME

Chez les sujets atteints de rhinite allergique, la réponse inflammatoire ayant lieu au niveau de la muqueuse nasale est en grande partie une conséquence de l'activation de cellules inflammatoires par l'intermédiaire d'IgE. La présence de ces IgE au niveau tissulaire a été attribuée à l'infiltration de cellules inflammatoires fixant l'IgE et/ou les cellules plasmatiques productrices d'IgE. L'IL-4 et l'IL-13 induisent les cellules B à transcrire le gène précoce ε et à s'engager dans la recombinaison aléatoire isotypique vers l'IgE. Les lymphocytes B ainsi que d'IL-4 et d'IL-13 été démontrée au niveau de la muqueuse nasale de patients atteints de rhinite allergique, suggèrant qu'une recombinaison aléatoire isotypique vers l'IgE pourrait avoir lieu localement dans le tissu. Ici, nous avons émis l'hypothèse que les cellules B résidentes de la muqueuse nasale pourrer être amenées à se convertir en cellules productrices d'IgE. Des sondes d'ADNc chargées de reconnaître spécifiquement les ARNm codant pour l'Iε (gène précoce nécessaire à la recombinaison isotypique), le Cε (transcrit mature) ainsi que l'IL-4 et l'IL-13. Comme il a été mis en évidence que les stéroïdes réduisaient le nombre de cellules exprimant les ARNm codant pour l'IL-4 et l'IL-13, les effets d'un prétraitement avec un stéroïde local (le propionate de fluticasone) sur la synthèse de l'ARNm codant pour ε.

Les patients étaient tous des individus présentant une rhinite allergique saisonnière. Initialement, les biopsies de muqueuse nasale ont été obtenues en dehors de la saison pollinique et puis après une provocation allergènique *in vivo*. Ultèrieurement, le tissu nasal a été prélevé des patients avant et pendant la saison pollinique. Afin, de confirmer une synthèse locale d'ARNm, nous avons reproduit *ex vivo* une provocation antigènique. La présence de cellules B dans le tissu nasal a été confirmée par

immunocytochimie (ICC). Par hybridation *in situ* (HIS), une augmentation du nombre d'ARNm codant pour le Cε et l'IL-4, ainsi que la présence de cellules positives pour l'ARNm de l'Iε ont pu être observées dans les sections de tissu nasal obtenu de patients traités par un placebo, à la suite d'une provocation allergènique *in vivo* ou naturelle, et que cette augmentation n'a pas été constatée dans le tissu obtenu des patients traités avec le propionate de fluticasone. Une exposition *ex vivo* à l'allergène entraîne des résultats similaires, à savoir une augmentation du nombre de cellules exprimant les ARNm codant pour le Cε, Iε, l'IL-4 et l'IL-13, dans les tissus stimulés par l'allergène par comparaison aux tissus non-stimulés. En combinant les techniques ICC/HIS, nous avons démontré que 37% des cellules positives pour le CD20 le sont aussi pour le Cε, contre 32% pour le Iε. De plus, par double (HIS), la présence de cellules $C\varepsilon^{+}/I\varepsilon^{-}$ a été mise en évidence, confirmant l'existence d'un ARNm codant pour l'ε mature. Des expériences de colocalisation illustrent également que la production locale d'IL-4 est attribuée presque entièrement aux cellules T (≅70%) et mastocytes (≅32%) alors que la capacité de ces types cellulaires à produire de l'IL-13 est moindre (≅44%, ≅18%).

En résumé, les données exposées dans cette thèse mettent en évidence une synthèse locale des transcrits précoce et mature d'ε ainsi que de ceux codant pour les facteurs inducteurs de la recombinaison aléatoire isotypique vers l'IgE, à savoir l'IL-4 et l'IL-13, et de plus, le prétraitement avec un stéroïde local engendre l'inhibition de ces augmentations. Par conséquent, ces résultats suggèrent que la muqueuse nasale peut être un site primaire pour reglé la reponse d'antigène et que met l'accent sur l'importance du diagnostique et des stratégies thérapeutiques visant à cibler la réponse inflammatoire locale.

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Chapter 1

1.0 INTRODUCTION

1.1 Allergy

The term *allergy* was first introduced by Von Pirquet in 1906 to describe one's 'changed reactivity' to foreign antigen, irrespective of whether it resulted in a protective or hypersensitive immune response [von Pirquet et al, 1963; Kay et al, 1997]. In more recent times, this term has been increasingly used to describe the latter of the two scenarios, specifically the Type I hypersensitive response mediated by the production of allergen-specific IgE and associated with allergic diseases such as atopic dermatitis, asthma and rhinitis. In the western world allergic disease is common, affecting some 15-20% of the North American population. Although there is no clear Canadian data, it does appear that the prevalence of allergic skin and airway disease has increased worldwide [Burney et al, 1990; Ninan et al, 1992; Peat et al, 1994; Aberg et al, 1995; Magnus et al, 1997].

What is causing this rise in prevalence? There are no real answers as yet, although a number of possibilities have been suggested. Since the Industrial Revolution in the mid 1700s air pollution has been increasing, to the point that it is now a major environmental and medical problem. It is considered to have a particular impact on the manifestation of allergic disease [Emanuel et al, 1988; Corbo et al, 1993; Rusznak et

al, 1996]; there is direct evidence that diesel exhaust particles act as an adjuvant for allergen-induced production of IgE [Diaz-Sanchez et al, 1994a]. Other irritants, such as cigarette smoke, are considered to increase the susceptibility of respiratory epithelium to allergen [Rusznak et al, 1997] and the prevalence of nasal symptoms [Bascom et al, 1991], while modern items such as air conditioning and carpeting have been suggested to enhance the level of indoor allergen one is routinely exposed to. There are also reports that encounter with some viruses may exert a protective effect against the development of allergy. In Guinea-Bissau, West Africa, measles infection in early life was associated with a large reduction in skin-prick positivity to housedust mite after a 14 year follow up [Shaheen et al, 1996] and in a study of Italian military students, allergic disease was significantly less common in those who were sero-positive than sero-negative for hepatitis A [Matricardi et al, 1997]. Furthermore, this rise in prevalence has occurred along side advances in the treatment and circumvention of viral infections, such as vaccines and anti-viral medications. From this body of work, it could be speculated that it is the ever-'progressing' society in which we live that may be, at least partially, responsible for this issue of increased prevalence in allergic disease.

1.2 Atopy and sensitization

Acquired immune responses are mediated through the cellular and/or humoral immune system. Cellular immunity is concerned mainly with the clearance of pathogens such as bacteria and utilizes macrophages, T cells and their derived cytokines to perform this function. Humoral immune responses also rely heavily on T cells and their associated

products, however, the main effector cell is the B lymphocyte and their production of antigen-specific immunoglobulins [Abbas, 1994a]. In normal individuals, antigen activation of B cells results primarily in the production of IgM and IgG [Brostoff et al, 1996]. In some cases, however, B cells respond to antigen by producing quantities of specific IgE, commonly detected by radioallergosorbent tests (RAST) and skin-prick testing. The synthesis of high levels of allergen-specific IgE is, in itself, indicative of an aberration in the normal immune response. This condition is referred to as *atopy*, derived from the Greek *atopia* for strangeness [Cohen et al, 1992; Kay et al, 1997]. However, many atopic individuals do not manifest symptoms of any allergic disease.

In those that do develop allergic responses, several encounters with the antigen is usually necessary before one exhibits full blown symptoms. This is attributed to the fact that the inflammatory cells must be primed in order to respond to antigen. Initial exposures induce production of IgE, which binds to Fcε receptors (FcεR) on inflammatory cells. Once a baseline level of IgE+ cells is attained, subsequent exposures to antigen will be associated with an immediate allergic response [Naclerio et al, 1998]. These initial exposures and the process of priming the inflammatory cells for response to antigen is referred to as *sensitization.*

Despite an increasingly large number of studies dedicated to describing the characteristic features of the various forms of allergy, the fundamental defect(s) involved in the excessive production of specific IgE remains elusive. There is evidence that atopy may be attributed to genetic predisposition. Linkage analysis studies have implicated a number of potential sites, including HLA molecules [Levine et al, 1972;

Cookson et al, 1989] and the cytokine gene cluster on chromosome 5 [Marsh et al, 1994; Noguchi et al, 1997], particularly IL-9 [Doull et al, 1996]. Nonetheless, what distinguishes an atopic individual from one that develops allergic symptomatology is still unknown. Furthermore, although it appears that elements of both heredity and environment are intergral to the manifestation of allergic disease, it is not clear why one may suffer from organ-specific responses.

1.3 Immediate type hypersensitivity

Type I or immediate hypersensitivity is one of the four hypersensitive immune responses and is characterized by its rapid onset, within minutes of exposure [Kay et al, 1997]. The inflammatory characteristics of the immediate hypersensitive response have been largely determined through the observation of changes resulting from acute antigen challenge of allergic individuals, using cutaneous intra-dermal [Tsicopoulos et al, 1994] and intra-nasal challenge with specific allergen [Naclerio et al, 1985; Durham et al, 1992; Varney et al, 1992]. Within the nose there is a biphasic response, designated the early and late phase. The early phase occurs within minutes of allergen exposure and is considered a direct consequence of allergen cross-linkage of IgE molecules bound on mast cells and other FcεR-expressing cells [Naclerio et al, 1997]. Once these cells are activated, they degranulate and release an array of pro-inflammatory mediators, their bioactivity inducing the development of clinical symptoms [Naclerio et al, 1983; 1990]. The late phase is observed between 1 to 24 hours following allergen exposure and is attributed primarily to the infiltration of T

cells and eosinophils and the release of their mediators, paritcularly Th2 cytokines [Varney et al, 1992; Durham et al, 1992].

1.4 Allergic disease of the airways

Many individuals experience immediate hypersensitivity to antigen in both the upper and lower airways. Nasal symptoms have been reported to occur in as much as 78% of asthmatic patients [Blair et al, 1977; Pedersen et al, 1983], while it is estimated that up to 38% of those with seasonal allergic rhinitis have asthma [Aberg et al, 1989]. In some cases rhinitis may be a predisposing factor for asthma, since non-asthmatic individuals with allergic nasal symptoms were seen to develop asthma 3 times more often than those without rhinitis, over a 23 year period [Settipane et al, 1994]. Although this work demonstrates that these two allergic respiratory disorders commonly co-exist, a fair number of patients appear to exhibit symptoms restricted to one compartment or the other. The factors responsible for this are poorly understood. It has been suggested that there may be an allergic continuum, by which sensitivity commences in one site and progress along the airway tract. This does not explain all cases, since there are individuals who do not develop both conditions. There is also the possibility that individuals with *only* allergic rhinitis or *only* asthma may have a more prominent local rather than the supposed systemic response, accounting for the manifestation of site-restricted symptoms.

1.5 Rhinitis

Rhinitis is defined as an inflammation of the nasal mucosa accompanied by periods of sneezing, puritis, congestion and nasal discharge [Howarth et al, 1989; Parikh et al, 1997]. The onset of a rhinitic event can be triggered by different aero-antigens, including viral and/or bacterial infection as well as sensitivity to specific allergens. As such, rhinitis can be classified according to its allergic or non-allergic nature.

1.5a Non-allergic rhinitis

There are many factors that induce the symptoms of rhinitis. Infectious rhinitis, frequently referred to as the 'the common cold', is accompanied by cloudy (white, yellow or green) nasal secretions and sneezing and is attributed mainly to viral rather than bacterial infection [Bardin et al, 1994]. Bradykinin and certain cytokines have been implicated in the pathophysiology of this form of rhinitis, since they have been detected within nasal secretions of patients with rhinovirus upper respiratory infection [Gwaltney et al, 1995; Igarashi et al, 1993]. Furthermore, in some individuals cold, dry air can induce the symptoms of rhinitis with secretions of significantly high osmolarity [Togias et al, 1988; 1990].

1.5b Allergic rhinitis

Although viral infections of the upper respiratory tract are the most common cause of *nasal-itis*, allergic rhinitis is highly prevalent. It is characterized by similar symptoms of sneezing, puritis and congestion, however, allergic rhinitis is accompanied by clear watery secretions. It manifests as either a perennial or seasonal condition. Perennial allergic rhinitis arises from allergens like mold and dust mite dander, which are present

in the air and perpetuate allergic symptoms year round. Allergens, such as grass pollens, trees and weeds are in the air for only a discrete period of time during the plant's pollination period and therefore induce transient nasal symptoms. The major allergens implicated in seasonal rhinitis are grass pollens, present in May through to July [Esch et al, 1999], and ragweed, in August to September [Mohapatra et al, 1999].

1.5c Nasal provocation

Provocation of the nasal mucosa of sensitized individuals with specific allergen creates a model of allergic rhinitis. The induced symptoms include sneezing, puritis, rhinorrhea and nasal congestion, just as in naturally occurring rhinitis. There are many mediators associated with these symptoms, including histamine, the cysteinyl leukotrienes (LTC_4, D_4 and E_4) and PGD_2. Analysis of nasal lavage [Naclerio et al, 1983], nasal secretions and nasal biopsy [Winther et al, 1987; Fokkens et al; 1989, Stoop et al, 1989; Hameleers et al, 1989; Hellquist et al, 1991; 1992; Ying et al, 1991] has enabled investigation of mediator release into the fluid phase and their presence within the tissue. Kinetic studies have revealed that sneezing and nasal secretion peaks within the first 2 minutes following acute antigen challenge, paralleling histamine release [Iliopoulos et al, 1990; Naclerio et al, 1997], and reoccur during the late phase response. This persistance is considered to be due primarily to increased vascular permeability and glandular secretion induced by the action of the cysLTs [Okuda et al, 1988; Rachelefsky et al, 1997] and PGD_2 [Doyle et al, 1990; Naclerio et al, 1993] on sensory nerve fibers. Nasal congestion and blockage are a result of increased resistance to airflow, attributed to vascular obstruction [Howarth et al, 1997]. Histamine, PGD_2,

cysLT and the neurokinins have been shown to cause vasodilation and are therefore implicated in sinusoid engorgement of the turbinates [Devillier et al, 1988; White et al, 1992; Howarth et al, 1997]. As such, nasal blockage is seen early, but it increases gradually and is most prominent in the late phase.

1.6 The nasal mucosa

Nasal tissue is part of the respiratory mucosal tract and is lined by an epithelial layer resting on a continuous basement membrane, with an underlying submucosal area composed of blood vessels, nerves, glands, lymphatics, extracelluar matrix, interstitial and inflammatory cells. The pathogenesis of allergic rhinitis is largely characterized by dysfunction of one or more of these components. As such, an understanding of this compartment under normal conditions is important for studying the abberations that occur as a result of allergy.

Healthy nasal mucosa is lined by pseudostratified columnar epithelium consisting of ciliated and non-ciliated columnar epithelial cells [Halama et al, 1990], goblet cells [Tos et al, 1983] and basal cells [Eccles et al, 1995]. Ciliated columnar cells start to appear just behind the front edge of the inferior turbinate and increase moving posteriorly in the nose [Mygind et al, 1997]. All four cell types rest on a basement membrane composed of collagen types I, III and IV fibrils [Sanai et al, 1999], however, not all cells reach the luminal surface, hence the illusion of a stratified layer.

The nose is one of the most vascular organs of the body, with a total blood flow/cm^3 that exceeds the muscle, brain and liver [Drettner et al, 1974]. The maxillary artery is the major source of blood flow to the nasal mucosa [Burham et al, 1935]. The

arterioles have no elastic basement membrane and as such the underlying smooth muscle cells are readily influenced by inflammatory mediators [Cauna et al, 1970a; 1970b]. The capillaries are situated close to the glands [Cauna et al, 1969; 1970a; 1970b] and the cavernous sinusoids are localized to the basal part of the submucosa; especially prominent in the turbinates.

The nasal mucosa is innervated by both sensory and autonomic nerves. The sensory afferents are from the trigeminal nerve, the parasympathetic fibers run within the facial nerve and the sympathetic fibers are derived from the vidian nerve [Eccles et al, 1973]. Afferent impulses progate via the sensory fibers to the central nervous system, giving rise to the characteristic sneezing, glandular secretion and changes in blood flow [Eccles et al, 1973]. Blood vessels are dilated and constricted by stimulation of the parasympathetic and sympathetic nerves, respectively. The sympathetic fibers are especially rich in the wall of the sinusoids, which account for a large part of blood volume to the turbinates, and changes in this innervation can cause considerable and rapid alteration in the thickness of the mucosa and consequently in the degree of airway resistance [Olsson et al, 1986].

The submucosal layer consists of serous and seromucous glands, at a ratio of 8:1 [Widdicombe et al, 1982]. These glands, along with epithelial goblet cells, synthesize the mucous which overlies the epithelium and provides anti-microbial function as well as transport for foreign particles via mucocilliary clearance [Kaliner et al, 1991]. They are under parasympathetic innervation, stimulation of which causes the characteristic watery nasal secretion [Eccles et al, 1973].

Lymphatic vessels pervade the nasal mucosa soaking up lymph and foreign antigen. Lymph drains from these to consecutively larger vessels, to the adenoids and tonsils within the ring of Waldeyer and local draining lymph nodes [Ganzer et al, 1988].

The resident inflammatory cells within the nasal mucosa are comprised primarily of lymphocytes, macrophages and mast cells. The ratio of lymphocytes to macrophage has been reported to be 10:1 [Winther et al, 1987] and to mast cells 6:1 [Igarashi et al, 1995]. Within the lymphocyte subset, there are 3 times as many T cells as B cells, and the CD8+ are 2/5ths that of CD4+ T cells [Winther et al, 1987].

1.7 Inflammation of the nasal mucosa

Within allergic nasal mucosa the cells and mediators are altered in comparison to normal nasal mucosa. There are increased numbers of CD4+ T cells, mast cells, basophils, eosinophils and antigen presenting cells. These cells are considered to infiltrate the target organ through site-specific endothelial expression of adhesion molecules as well as a complex array of chemokines, which create a chemotactic gradient by which the cells transmigrate the endothelium. Once within the nasal mucosa these cells serve as potent sources of pro-allergic mediators such as histamines, eicosinoids and cytokines, which work together to mediate and enhance the allergic response.

1.7a Epithelium

Like the skin, the role of the respiratory epithelium has long been considered as a barrier for protecting the inner mucosa from inspired antigens. The epithelial layer of allergic nasal mucosa is characterized by areas of focal denudation and squamous cell

metaplasia, which are improved following intra-nasal steroid treatment [Minshall et al, 1998]. While it seems likely that diminished epithelial integrity may facilitate antigen passage and entry into the submucosa, definitive evidence of this is yet to be demonstrated. More recently, however, epithelial cells have been shown to express MHC class II [Godthelp et al, 1996] and to play an active role in the initiation of the allergic response through antigen presentation [Salik et al, 1999] as well as production of a number of allergic mediators, such as cytokines and chemokines [Mullol et al, 1995; Hamilos et al, 1998; Minshall et al, 1997; Laberge et al, 1997; Christodoulopoulos et al, 1999].

1.7b Mast cells

Mast cells are identified within the tissue by their production of the serine endopeptidases, tryptase [Schwartz et al, 1981] and/or chymase [Schechter et al, 1983]. In fact, they have been subtyped according to their expression of these enzymes, those that produce both tryptase and chymase (Mtc) and those that produce only tryptase (Mt) [Otsuka et al, 1985; Church et al, 1997]. The majority of mast cells within allergic nasal mucosa are Mt within the epithelial layer, while the Mtc are found in close proximity to the nerves and blood vessels [Bentley et al, 1992].

Although the total number of mast cells does not change during the allergy season, a higher proportion are observed just beneath or within the epithelial layer [Lozewicz et al, 1990; Bentley et al, 1992; Bradding et al, 1993]. Elevated tryptase levels have been detected in nasal lavage from symptomatic compared to asymptomatic allergic rhinitics and normal controls [Wilson et al, 1998]. Mast cells express the high affinity

receptor (FcεRI) for IgE [Metzger et al, 1992; Pawankar et al, 1997]. Upon allergen exposure IgE molecules are cross-linked by antigen causing mast cell activation, degranulation, and immediate release of stored histamine [Uvnas et al, 1970; Naclerio et al, 1983], PGD_2 [Dahlen et al, 1986; Benyon et al, 1987], IL-4 [Bradding et al, 1992], TNF-α [Ohkawara et al, 1992; Gordon et al, 1991] and the appearance of message for IL-5, GM-CSF and IL-13 within 2 hours [Bressler et al, 1997; Pawankar et al, 1997]. Mast cells also express MHC class II [Dimitriadou et al, 1998; Poncet et al, 1999] and CD40L [Gauchat et al, 1993]. Steroids reduce the number of tissue mast cells [Rak et al, 1994], considered a consequence of inhibiting production of mast cell growth and survival factors, stem cell factor in particular [Mekori et al, 1993; Kim et al, 1997; Finotto et al, 1997].

1.7c Basophils

Unlike mast cells, basophils are not considered a normal component of the inflammatory cell complement of the nasal mucosa. They are circulating cells that pass from the blood to the tissue following allergen exposure [Schroeder et al, 1997]. As such, they are increased within the peripheral blood as well as nasal secretions of allergic rhinitics compared to controls [Walden et al, 1988, Bascom et al, 1988; 1988b; Naclerio et al, 1988]. Basophils are also a source of histamine [Valenta et al, 1993] and LTC_4 [Howarth et al, 1989]. They express FcεRI [Metzger et al, 1992; Sihra et al, 1997], synthesize IL-4 and IL-13 following IgE receptor stimulation [Brunner et al, 1993; Schroeder et al, 1994; Li et al, 1996; Gibbs et al, 1996] and constitutively express CD40L [Gauchat et al, 1993]. The relative importance of this

cell population to cytokine synthesis within the tissue is, however, uncertain as IL-4 has been co-localized predominantly to T cells and mast cells in nasal biopsies [Bradding et al, 1993; Ying et al, 1994].

1.7d Eosinophils

Like basophils, eosinophils are rarely found within the nasal muocosa under normal circumstances. These cells are characterized by the expression of cytotoxic granular proteins, including major basic protein (MBP) and eosinophil cationic protein (ECP) [Peters et al, 1986; Bascom et al, 1989]. MBP, in particular, has been reported to damage respiratory epithelium [Hisamatsu et al, 1990] and to induce degranulation of other inflammatory cells [Thomas et al, 1989]. Eosinophils are increased in number within the nasal mucosa of individuals with naturally occuring active rhinitis [Bentley et al, 1992; Bradding et al, 1993] as well as following acute antigen exposure [Bascom et al, 1988; Varney et al, 1992; Masuyama et al, 1998]. Eosinophils express both the high and low affinity receptor for IgE (FcεRI and RII) [Jouault et al, 1988; Soussi Gounni et al, 1994], MHC II molecules [Hansel et al, 1992; Weller et al, 1993] and CD40L [Gauchat et al, 1995]. Subsequent to antigen crosslinkage, these cells release a collection of pro-allergic/inflammatory cytokines, including IL-4, IL-5, GM-CSF and TNF-α [Broide et al, 1992; Costa et al, 1993; Nonaka et al, 1995]. Topical corticosteroids reduce the number of eosinophils within allergic nasal mucosa and the presence of cationic proteins within lavage fluid [Togias et al, 1988; Holm et al, 1995].

1.7e T lymphocytes

The T lymphocyte is the most prominent inflammatory cell within the nasal mucosa [Winther et al, 1987; Naclerio et al, 1997]. As precursors, T cells move from the bone marrow to the thymus for maturation. The T cell antigen receptor (TCR) is non-covalently associated with cluster determinant (CD)3 proteins, together comprising the TCR complex. The TCR recognizes sequences of amino acids in the peptides presented by MHC molecules, while CD3 is responsible for signal transduction and cell activation. Expression of the co-receptors CD4 or CD8 divide the T cell population into two functionally distinct subsets. CD8+ T cells are associated with cellular immunity and recognize MHC class I associated antigen. CD4+ T cells are termed helper T cells (Th cells), are MHC class II restricted and involved mainly in humoral immune responses [Abbas, 1994b; 1994c; Lydyard et al, 1996]. The local environment in which CD4 T cells are activated influences them toward either the T helper 1 (Th1) or Th2 subtypes.

Th1 and Th2 subtypes were initially described by Mosmann in 1986 when murine CD4 T cells were classified on the basis of distinct cytokine expression patterns [Mosmann et al, 1986]. This work was seminal in guiding our understanding and subsequent subtyping of T cells. Th1 cells are considered to produce high levels of IFN-γ and IL-2 and are involved in delayed type hypersensitivity, while Th2 cells mainly produce IL-4, IL-5 and IL-13 and are associated with immediate type hypersensitivity, *i.e.* allergic responses [Abbas et al, 1996]. When naïve T cells are activated in the presence of IL-12 or IL-4, they take on a Th1 or Th2 phenotype,

respectively [Seder et al, 1992; Hsieh et al, 1993; Trinchieri et al, 1995; Abbas et al, 1996]. The IL-12 receptor (IL-12R) consists of a β_1 and β_2 subunit. IL-12Rβ_1 is expressed on both Th1 and Th2 cells, while IL-12Rβ_2 is exclusive to Th1 [Rogge et al, 1997]. The β_2 is now considered the pivotal point for Th1 and Th2 phenotype development, since IL-4 inhibits and IFN-γ induces its expression [Szabo et al, 1995; Szabo et al, 1997]. Additionally, a reciprocal inhibitory relationship has been shown to exist between IL-4 and IFN-γ [Rousset et al, 1991] themselves, which recent work indicates may be attributed to DNA methylation patterns [Bird et al, 1992; Agarwal et al, 1998; Takemoto et al, 1998] and cell division number [Gett et al, 1998; Bird et al, 1998]. Th0 T cells, producing IL-2, IL-4, IL-5 and IFN-γ, are the forerunner to the distinct Th1 and Th2 T cells. They are observed early after lymphocyte activation [Kelso et al, 1995], while the polarized Th1 and Th2 subtypes are predominantly observed in chronic disease states [Romagnani et al, 1994]. Although *in vitro* work has demonstrated that repeated stimulation induces irreversibly committed phenotypes [Perez et al, 1995; Sornasse et al, 1996; Murphy et al, 1996], it is not clear whether this polarization occurs *in vivo* [Mosmann et al, 1989; Kelso et al, 1995].

The total number of T lymphocytes is unchanged within the nasal mucosa of seasonal allergic rhinitics before compared to during the season [Bentley et al, 1992]. There are, however, more activated T cells (CD25+) [Hellquist et al, 1992], which appear to be mainly expressing CD4+ [Varney et al, 1992]. Furthermore, steroid pre-treatment inhibits allergen-induced increases in CD4+ and CD25+ T cells within nasal mucosa of allergic rhinitics [Rak et al, 1994].

While CD8+ T cells are prominent during viral infections [Hsia et al, 1989] and are primarily considered to mediate cellular immune responses [Abbas 1994c, Thompson et al, 1996], a population of these cells are present within allergic nasal mucosa [Wright et al, 1999]. Co-culture of CD8 T cells and B cells resulted in IL-4 associated production of IgE [Meissner et al, 1997], while a mouse model of viral challenge has demonstrated CD8 T cell production of IL-5 and tissue eosinophilia [Coyle et al, 1995]. As such, it is possible that these cells may also be involved in propagating the allergic response to allergen.

1.7f B lymphocytes

B lymphocytes are the central cell of the humoral response, owing mainly to their exclusive ability to synthesize antibodies. B cell maturity is attained within the bone marrow and signified by the surface expression of IgM (sIgM) and sIgD. Encounter with antigen prior to this stage will discontinue B cell development [Abbas, 1994d], which has been suggested to be attributed to developmental changes in transcription factor expression [Seyfert et al, 1990]. B lymphocytes recognize and respond to antigen using antigen-specific receptors; B cell receptors (BCR). The BCR is a complex composed of the signal transducing proteins Igα and Igβ, analogous to CD3 on T cells, and membrane-bound or surface Ig (sIg) for antigen recognition, *i.e.* B cells producing IgE express sIgE for the BCR complex [Abbas, 1994d].

B cells move out of the bone marrow and migrate to the secondary lymphoid organs for differentiation. *Naïve* B cells are sIgM+/sIgD+ and have not yet seen antigen. Following antigen stimulation, B cells either become *memory* B cells or

plasma cells. Memory B cells retain their cognitive abilities and circulate within the periphery. These cells are a highly important component of allergy, as they can survive for long periods of time in the absence of antigen and then provide a rapid source of high affinity antibody when stimulated. Plasma cells are terminally differentiated cells that produce massive amounts of Ig. They can reportedly house as much as 500 times more cytoplasmic Ig mRNA than non-secreting B cells [Abbas, 1994b]. These cells no longer have the ability to proliferate or isotype switch in response to antigenic stimulation and are relatively short lived [Ho et al, 1986; Esser et al, 1990; Feuillard et al, 1995]. As such, they are considered at least one of the mechanims by which the immune system controls B cell clonal expansion. CD20 is a pan B cell marker that recognizes naïve, resting and memory B cells and is therefore often used for their identification in a mixed cell population [Zola et al, 1987]. Memory B cells are CD20+/CD38- [Pascual et al, 1994], while plasma cells are CD20-CD38+ [Harada et al, 1993; Arpin et al, 1995; Arpin et al, 1997]. CD23 (low affinity IgE receptor) has been used as a marker for B cells that have yet undergone isotype switching [Kikutani et al, 1986; Kehry et al, 1989; Pene et al, 1988a; Bonnefoy et al, 1996]. Furthermore, CD27 has been suggested as a marker for memory B cells [Klein et al, 1998].

The number of B lymphocytes within the nasal mucosa is substantially less than T lymphocytes [Winther et al, 1987; Davidsson et al, 1994]. Typically they are seen to form aggregates within the submucosa [Stoop et al, 1989] and are rarely intra-epithelial [Hameleers et al, 1989]. B cell number within the nasal mucosa does not appear to change following antigen challenge [Davidsson et al, 1994].

1.7g Antigen presenting cells

The defining features of an antigen presenting cell (APC) are the ability to *a)* bind and/or take up antigen and *b)* present the processed antigenic peptide to T cells via MHC molecules. The APC must also express accessory molecules which co-stimulate the T cell's response. Since most mammalian cells endocytose and process protein antigens, the critical factors for antigen presentation are expression of MHC class II and co-stimulatory molecules [Abbas, 1994e, Feldmann, 1996].

The cells most commonly considered APC are macrophages, dendritic cells and B cells. Macrophages take up antigen by phagocytosis as well as by Ig:Fc receptor (FcR) complexes and they express MHC class II molecules following activation [Feldmann, 1996]. Dendritic cells use macropinocytosis as well as Ig:FcR complexes to detect antigen [Sallusto et al, 1995], particularly FcεRs [Bieber et al, 1989; Wang et al, 1992; Maurer et al, 1996]. Unlike macrophages, however, dendritic cells constitutively express MHC class II molecules and as such are considered to be more efficient antigen presenters [Feldmann, 1996]. B cells also function as APC, binding antigen either by the BCR or Ig:FcR complexes [Feldmann, 1996]. Unlike macrophages and dendritic cells, they do not have phagocytic or pinocytic capablilites. As such, B cells confer specific high affinity antigen binding. Although B cell expression of MHC II is not constitutive, like the dendritic cell, their mechanism for antigen detection makes them important for presentation in cases of low antigen concentration.

Within the nasal mucosa there are many cell types which may function as APC, however, which one(s) are primarily responsible during the allergic response to antigen

is yet to be determined. Macrophages seem to be a less likely candidate, since antigen presentation by these cells reportedly induces CD4+ T cells to produce IFN-γ [Secrist et al, 1995]. It does appear, though, that the dendritic cell may play an important role in the presentation of antigen leading to an allergic nasal response. These cells are increased within the nasal mucosa of patients with seasonal allergic rhinitis [Fokkens et al, 1989] and are further elevated in number following antigen challenge [Godthelp et al, 1996]. Langerhans cells, considered to derive from the dendritic cell lineage, within allergic nasal tissue have been observed to be both IgE and MHC class II+ [Godthelp et al, 1996]. Furthermore, *in vitro* these cells are reported to induce stable IL-4-producing T cell lines in response to antigen stimulation [Hauser et al, 1989]. In similar experiments, B cells have also been shown to stimulate IL-4 synthesis from CD4+ T cells when cultured with antigen [Secrist et al, 1995]. Furthermore, there are a number of cell types within the nasal mucosa not classically considered APC, that nonetheless seem capable of antigen uptake and expression of MHC class II molecules. These include structural cells such as the epithelium [Nonaka et al, 1996] and inflammatory cells like mast cells [Metzger et al, 1992; Pawankar et al, 1997; Dimitriadou et al, 1998; Poncet et al, 1999] and eosinophils [Jouault et al, 1988; Weller et al, 1993; Soussi Gounni et al, 1994]. The relative importance of these cell types in antigen presentation leading to the development of an 'allergy conducive' milieu has not yet been determined.

1.8 Cytokines

1.8a General overview

Cytokines are low molecular weight proteins that are important mediators of immune responses. Originally, they were thought to be exclusively leukocyte-derived, however, it is now clear that most cell types produce one or more cytokines. Aside from IL-1β [Hazuda et al, 1989], IL-16 [Baier et al, 1997; Zhang et al, 1998] and IL-18 [Okamura et al, 1995], which are synthesized as precursor proteins that require extra-cellular proteases for activation, most cytokines are produced in fully active form. Unlike hormones, which travel through the blood and act at distant sites, cytokines are generally short range mediators that operate through paracrine and/or autocrine inter-cellular signals. This has been attributed, at least partially, to the short half life of their mRNA transcripts [Lindstein et al, 1989], a consequence of intracytoplasmic protein binding to tandem AU sequences in the 3' untranslated region of cytokine mRNA, targeting the transcript for degradation [Bohjanen et al, 1991; Rajagopalan et al, 1994]. Cytokines are pleiotropic molecules and as such are considered as a network, with the function of one commonly overlapping that of another [Paul et al, 1989].

1.8b The Jak-STAT pathway

The majority of cytokines act through specific membrane-bound heter- or homo-dimeric receptors which lack intrinsic kinase activity and rely on the activation and recruitment of intracellular tyrosine kinase proteins [Kishimoto et al, 1994; Pugh-Humphreys et al, 1998]. The most well described are the Janus kinases (JAK), named after the two-faced Roman god of portals, since they have tandem kinase domains

within their structure. JAK-related transcription factors are termed 'signal transducers and activators of transcription' (STATs). Upon ligand binding and dimerization of cytokine receptor subunits, the JAK kinases induce tyrosine phophorylation of the receptor, creating docking sites for the associated STATs. Receptor binding phosphorylates these STATs. They are then released from the receptor, dimerize and translocate to the nucleus. STAT-STAT dimers bind their respective DNA response elements and thereby initiate cytokine-directed gene transcription [Darnell et al, 1994; Leonard et al, 1998; Pugh-Humphreys et al, 1998].

1.8c Th1 and Th2 cytokines

In 1986, Mosmann *et al.* observed that mouse helper T cells cultured with particular antigens consistently expressed distinct cytokine profiles, referred to as Th1 and Th2 cells [Mosmann et al, 1986]. The Th1 cells expressed IL-2 and IFN-γ, while the Th2 cells expressed IL-4 and IL-5 [Cherwinski et al, 1987]. While there is debate over whether distinct Th1 and Th2 cells exist *in vivo* [Kelso et al, 1995; Mosmann et al, 1996], cytokines are now categorized according to this paradigm. In humans the Th1 cytokines are considered IL-2, IFN-γ and IL-12, even though the latter is not produced by T cells, and to mediate diseases such as microbacterial tuberculosis [Taha et al, 1997] and sarcoidosis [Minshall et al, 1997a]. The primary Th2 cytokines are IL-4 and IL-5 which mediate the allergic response to specific antigen [Durham et al, 1992; Del Prete et al, 1993]. There are, however, a number of cytokines, including IL-3, IL-10, IL-13, GM-CSF and TNF-α that are expressed during both Th1- and Th2-mediated immune responses [Mosmann et al, 1996]. As such, both the classic Th2

cytokines as well as those with a more promiscuous expression profile are implicated in the allergic response to antigen (Table 1.1).

Table 1.1 *Th1 and Th2 cytokines*

Response Type	Affiliated cytokines	Mediate Disease	References
Th1	IL-2,IFN-γ, IL-12, IL10, TNF-α, IL-3, GM-CSF	Tuberculosis Sarcoidosis	Mosmann 1986, 1996 Cherwinski 1987, Tsicopoulos 1994 Taha 1997, Minshall 1997
Th2	IL-4, IL-5, IL-13,IL-9, IL-10,IL-3, GM-CSF	Allergic rhinitis Asthma	Ying 1991, 1993, 1994 Durham 1992 Robinson 1996 Ghaffar 1997

IL-3, IL-5 and GM-CSF are most prominently characterized as hematopoietic factors, with particular importance for basophil and eosinophil differentiation [Clutterbuck et al, 1989; Denburg et al, 1991; Shalit et al, 1995]. Both IL-5 and GM-CSF enhance eosinophil survival, delaying the onset of apoptosis [Ochiai et al, 1997], while IL-5 has also been shown to induce IgA secretion from B cells [Sonoda et al, 1992]. IL-9 is also a Th2 cytokine that stimulates T cell proliferation [van Snick et al, 1989; Houssiau et al, 1993], mast cell growth [Hultner et al, 1990] and has been linked to serum IgE concentrations [Doull et al, 1996]. TNF-α, although mainly considered a pro-inflammatory cytokine, also activates eosinophils [Hossain et al, 1996; Luttmann et al, 1999] and facilitates immunoglobulin synthesis by B cells [Gauchat et al, 1992]. IL-10 is recognized for its immuno-suppressive effects, such as inhibiting cytokine production from Th1 cells [Fiorentino et al, 1991]. Moreover, it is a potent stimulator of B cells, inducing their proliferation and plasma cell differentiation [Rousset et al, 1992, Jeannin et al, 1998a]. Together IL-4, IL-13 and TNF-α mediate the infiltration of inflammatory cells such as eosinophils and T cells by upregulating expression of the adhesion molecule vascular cell adhesion molecule (VCAM-1) [Schleimer et al, 1992; Bochner et al, 1995; Thornhill et al, 1991], important for the transmigration of these cells through the endothelium and entry into the tissue [Wardlaw et al, 1996].

In addition to adhesion molecules, an integral component of the Th2 cytokine-mediated allergic response to antigen are the effects of the cytokine sub-family of

molecules called chemokines. These are low molecular weight mediators that have chemoattractant activity for inflammatory cells. They are catagorized according to the positioning of cysteine residues (C) within their amino acid sequence [Baggiolini et al, 1997]. To date, four families of chemokines have been described: C, CC, CXC and CXXXC. These mediators act by binding to specific G-protein-coupled cell-surface receptors on target cells [Murphy et al, 1994; Premack et al, 1996]. The CC chemokines, such as eotaxin, MCP (monocyte chemotactant protein)-4 and RANTES (regulated-on-activation normal T cell - expressed and secreted) are considered to be the most potent chemoattractants for eosinophils and T lymphocytes [Schall et al, 1988; Garcia-Zepeda et al, 1996a; 1996b; Kuna et al, 1998]. Furthermore, IL-4, IL-13 and TNF-α have been reported to increase the expression of these chemokines [Stellato et al, 1995; Garcia-Zepeda et al, 1996b; Li et al, 1999]. The combination of a chemokine gradient within the tissue and adhesion molecule expression on the luminal surface of the endothelium, both regulated by cytokines, appears to account for targeting a particular site for inflammatory cell infiltration [Luster et al, 1998].

The cytokines IL-5 [Pene et al, 1988a], IL-6 [Maggi et al, 1989], IL-7 [Jeannin et al, 1998b], IL-9 [Jeannin et al, 1998b] and TNF-α [Gauchat et al, 1992] have all been shown to enhance IgE production. However, these effects seem to be IL-4-dependent. IL-4 and IL-13 are considered the only cytokines with the ability to induce B cell committment to IgE production.

1.8d Properties of IL-4 and IL-13

IL-4 and IL-13 were identified in 1986 and 1992, respectively [Yokota et al, 1986; Minty et al, 1993; McKenzie et al, 1993a]. Human IL-4 and IL-13 have been mapped to chromosome 5 (band q23-31), together with a number of other related cytokine genes including IL-3, IL-5, IL-9, and GM-CSF [Asano et al, 1987; Morgan et al, 1992]. This loci is referred to as the 'IL-4 gene cluster' and spans approximately 3000 kb, however, the genes for IL-4 and IL-13 are in remarkably close proximity to one another, only 12 kb apart [Morgan et al, 1992; Smirnov et al, 1995].

1.9e Cellular sources and gene regulation of IL-4 and IL-13

Both IL-4 and IL-13 were initially discovered as T cell-derived factors [Yokota et al, 1986; Minty et al, 1993; McKenzie et al, 1993a]. Although the T lymphocyte is considered the major source of both these cytokines [Ying et al, 1994; Ghaffar et al, 1997], mast cells [Bradding et al, 1992; 1993; Pawankar et al, 1997], basophils [Brunner et al, 1993; Schroeder et al, 1994; Li et al, 1996; Shimizu et al, 1998] and eosinophils [Nonaka et al, 1995; Moqbel et al, 1995] have also been shown to contribute to their production. Unlike IL-4, however, IL-13 is also produced by macrophages [Hancock et al, 1998].

IL-4 and IL-13 gene expression is regulated by a number of transcription factors. Response elements for the 'nuclear factor of activated T cells' (NFAT) are present within the IL-4 and IL-13 promoters [Szabo et al, 1993; Dolganov et al, 1996]. This factor has been suggested to play a role in differential Th1/Th2 cytokine production, since its transcriptional activity was high in Th2 and low in Th1 T cell clones [Rincon

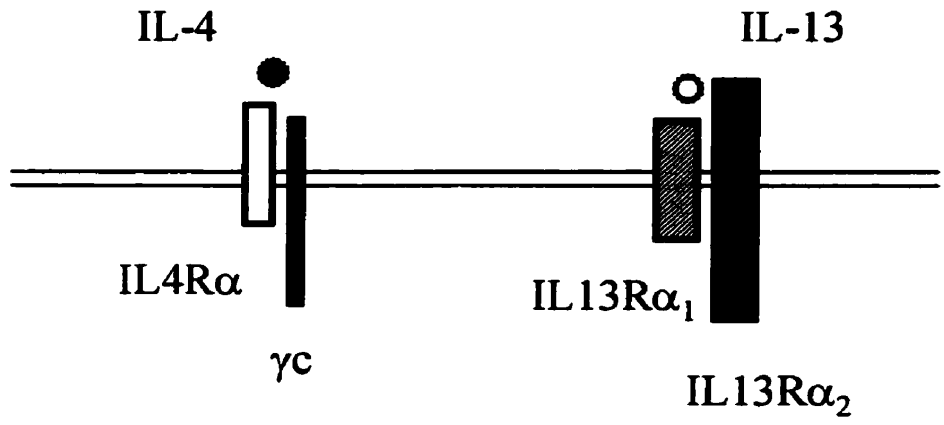
et al, 1997]. There are multiple allelic forms of the human IL-4 promoter. At least one of these has enhanced affinity for activating protein (AP)-1 [Song et al, 1996], required for optimal NFAT activity [Luo et al, 1996; McCaffrey et al, 1993; Jain et al, 1993], and may therefore account for the excessive production of IL-4 that characterizes allergy. Although originally considered to be T cell-restricted, NFAT is also implicated in IL-4 gene transcription in mast cells [Weiss et al, 1996]. Binding sites for the transcription factors STAT-6 [Curiel et al, 1997], c-maf [Ho et al, 1996] and GATA-3 [Smirnov et al, 1995; Rincon et al, 1997], of which the latter two are highly expressed in Th2 rather than Th1 clones [Zhang et al, 1997], have also been identified within the IL-4 promoter [Curiel et al, 1997; Ho et al, 1996; Zheng et al, 1997]. However, to date there is no real understanding as to the relative importance of these factors in regulating IL-4 and IL-13 gene expression.

1.8f IL-4 and IL-13 receptors

The IL-4 cytokine acts through a specific alpha chain receptor (IL-4Rα) [Galizzi et al, 1990; Idzerda et al, 1990] with an extracellular domain containing two motifs, 4 conserved cysteines and a WSXWS box; WS (tryptophan-serine) repeat spaced by one irrelevant (X) amino acid. This motif is characteristic of the cytokine receptor family [Miyajima et al, 1992]. There is a short hydrophobic domain that serves to retain the receptor within the membrane during secretion as well as a long cytoplasmic tail that associates with intracellular kinase proteins. IL-4Rα complexes with the IL-2Rγc chain, which increases the binding affinity of IL-4Rα by 2 to 3 fold [Russell et al, 1993]. The IL-13 receptor (IL-13R) exists as a complex of the IL-4Rα chain and one

of the IL-13 binding proteins, IL-13Rα_1 [Aman et al, 1996; Gauchat et al, 1997; Miloux et al, 1997] or IL-13Rα_2 [Caput et al, 1996]. Both subunits of the IL-13Rα are specific for IL-13, while IL-13Rα_1 on its own has lower affinity than IL-13Rα_2 [Aman et al, 1996; Caput et al, 1996; Pugh-Humphreys et al, 1998]. Upon dimerization with the IL-4Rα chain, both mediate high affinity binding. IL-13Rα_1 and IL-13Rα_2 have an additional STAT binding site within their cytoplasmic tail, which the IL-4Rα seems to lack [Aman et al, 1996; Caput et al, 1996; Gauchat et al, 1997; Miloux et al, 1997]. As such, IL-13 can act through an IL-4Rα-IL-13Rα complex or either IL-13Rα chain alone. Furthermore, in the absence of a functional γc chain, IL-4Rα-IL-13Rα can mediate IL-4 function [Obiri et al, 1995; Zurawski et al, 1995; Matthews et al, 1995; Izuhara et al, 1996]. IL-4 and IL-13 both activate the intracellular kinase JAK1 [Welham et al, 1995], leading to STAT-6 DNA binding [Hou et al, 1994]. IL-4 also activates JAK3, but this is through the γc subunit [Keegan et al, 1995; Welham et al, 1995] and is therefore not a function of IL-13 (Diagram 1.1). Nucleotide substitutions within the IL-4Rα chain have been associated with increased IL-4 signaling and function and therefore may mediate an increased sensitivity to IL-4 in cases of allergy [Hershey et al, 1997].

Receptors for both IL-4 and IL-13 have been identified on B cells [Yanagihara et al, 1995; Obiri et al, 1995; Ogata et al, 1998], while IL-4R are also observed on T cells [Zurawski et al, 1995; Obiri et al, 1995] and eosinophils [Dubois et al, 1998]. The number of cells expressing IL-4Rα within allergic nasal mucosal tissue from patients pre-treated with placebo is increased following antigen challenge, while pre-



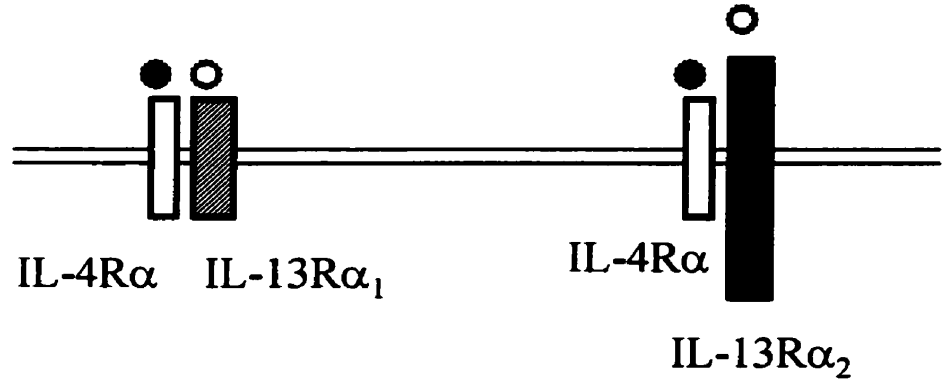


Diagram 1.1 Receptors for IL-4 and IL-13

treatment with corticosteroid reduces the number of cells expressing these receptors [Wright et al, 1999].

1.8g Effects of IL-4 and IL-13

IL-13 and IL-4 share a number of functions. This is attributed mainly to their overall 3D structures being highly similar [McKenzie et al, 1993b; Minty et al, 1993; Zurawski et al, 1993; Bamborough et al, 1994] as well as the fact that they share receptor subunits [Aman et al, 1996; Gauchat et al, 1997; Miloux et al, 1997]. While only IL-4 acts on T cells [Zurawski et al, 1994; de Waal Malefyt et al, 1995; Sornasse et al, 1996], both cytokines have multiple affects on B lymphocytes. They enhance B cell proliferation [Cocks et al, 1993], their expression of MHC II [Clark et al, 1989; Diu et al, 1990; Punnonen et al, 1993] and FcεRII [Clark et al, 1989; Punnonen et al, 1993; Park et al, 1996]. IL-4, but not IL-13 [Punnonen et al, 1993], enhances B cell expression of CD40 [Valle et al, 1989] as well as the co-stimulatory molecules B7 [Valle et al, 1991]. Most particularly, IL-4 and IL-13 induce B cells to undergo isotype switching in favor of IgE (Diagram 1.2).

1.8h Expression of IL-4 and IL-13 in allergic rhinitis

During the season, the amount of IL-4 protein is increased in nasal lavage fluid and there are more cells expressing IL-4 and IL-13 mRNA+ within the nasal mucosa of individuals with allergic rhinitis, compared to controls [Howarth et al, 1995; Benson et al, 1997; Pawankar et al, 1997]. In the absence of allergen exposure, there appear to

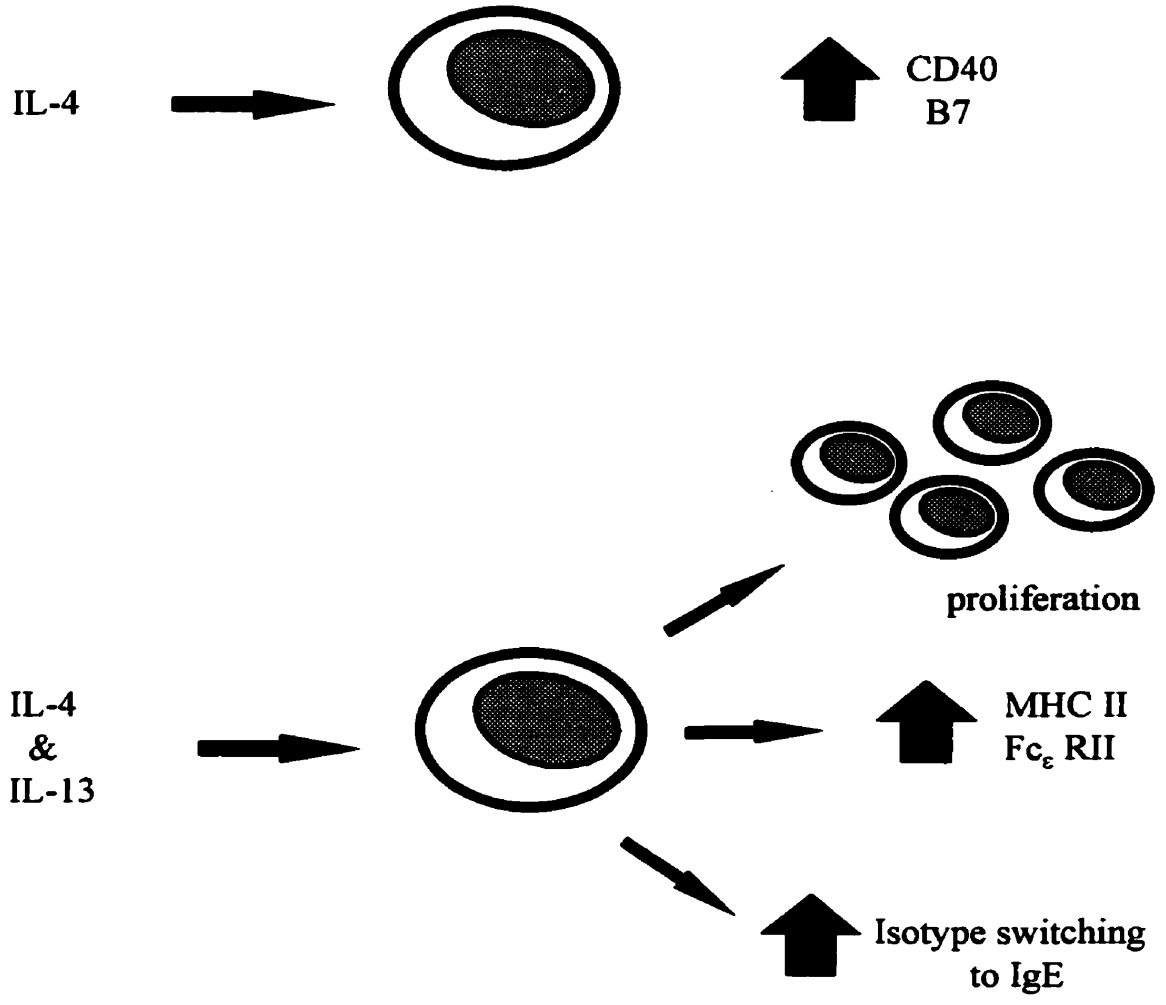


Diagram 1.2. Multiple effects of IL-4 and IL-13 on B lymphocytes

be more IL-4, but not IL-13, mRNA+ cells within allergic compared to normal nasal mucosa [Ghaffar et al, 1997]. Furthermore, peripheral blood mononuclear cells from seasonal allergic rhinitics produce IL-4 even in response to non-specific activation [Munoz-Bellido et al, 1998], indicating that inflammatory cells of allergic individuals are predisposed for IL-4 production. Allergen challenge studies demonstrate that the early increase in IL-4 protein is predominantly associated with mast cells and basophils [Wang et al, 1999], while at later time points the major source of IL-4 and IL-13 appears to be T cells [Ying et al, 1994; Ghaffar et al, 1997; Wang et al, 1999]. Kinetic studies have demonstrated that IL-13 is present up to 72 hours after activation of cultured T cells, whereas IL-4 was strongly decreased after 8 hours [de Waal Malefyt et al, 1995]. These findings suggest that although early mast cell release of IL-4 is considered crucial for initiation of the allergic response [Wang et al, 1999], IL-13 may play a more prominent role in the maintenance of chronic allergy. This coincides with *in vivo* findings demonstrating a much more pronounced upregulation of IL-13 than IL-4 mRNA 24 hours following allergen challenge [Ghaffar et al, 1997]. Furthermore, co-localization studies show that the expression of IL-13 is not always accompanied by the production of IL-4 [Ghaffar et al, 1997], consistent with the fact that IL-13 can be expressed by both Th1 and Th2 T cell phenotypes [de Waal Malefyt et al, 1995] as well as macrophages [Hancock et al, 1998].

1.9 Antigen Presentation and Germinal Center Formation

The majority of antigen presentation is considered to take place within the regional lymph nodes and spleen. Once inspired, antigen permeates the epithelial layer, enters

the nasal mucosa and travels, either in free form or bound to an APC, through the afferent lymphatics to be concentrated within the lymphoid tissues of Waldeyer's ring [Ganzer et al, 1988; Austyn et al, 1997], or the spleen [Abbas, 1994e].

1.9a Antigen presentation

Initial antigen presentation and B cell activation in lymphoid tissues occurs within the T cell rich zone, as the APC processes the antigen and presents it to T cells in association with MHC class II [Abbas, 1994e]. The APC expresses co-stimulatory molecules such as B7 [Caux et al, 1994a; Lenschow et al, 1994] that binds CD28, found constitutively on T cells [Caux et al, 1994a; Linsley et al, 1991; Jenkins et al, 1991]. These two signals, antigen presentation and co-stimulation, induce optimal T cell production of IL-2 and clonal expansion [Jenkins et al, 1991; Fraser et al, 1991] (Diagram 1.3). Since APC also express CD40, co-stimulation of T cells may occur through CD40L activation as well [Caux et al, 1994b]. Although the differential effects of these two ligand-receptor pairs (CD28:B7 and CD40L:CD40) on T cell activation is not clear, it is evident that in their absence antigen presentation results in T cell anergy [Schwartz et al, 1990; Jenkins et al, 1991; Griggs et al, 1996]. At this stage, the cytokine profile produced by T cells is thought to be determined. Th2 *vs.* Th1 cytokine production may be attributed to co-stimulation by B7-2, rather than B7-1 [Tsuyuki et al, 1997; Ranger et al, 1996], although this has not been confirmed in humans [Bashian et al, 1997]. Alternatively, antigen presentation by dendritic cells and B cells, rather than macrophages, may favor Th2 cytokine production [Hauser et al, 1989; Secrist et al, 1995; Gould et al, 1997].

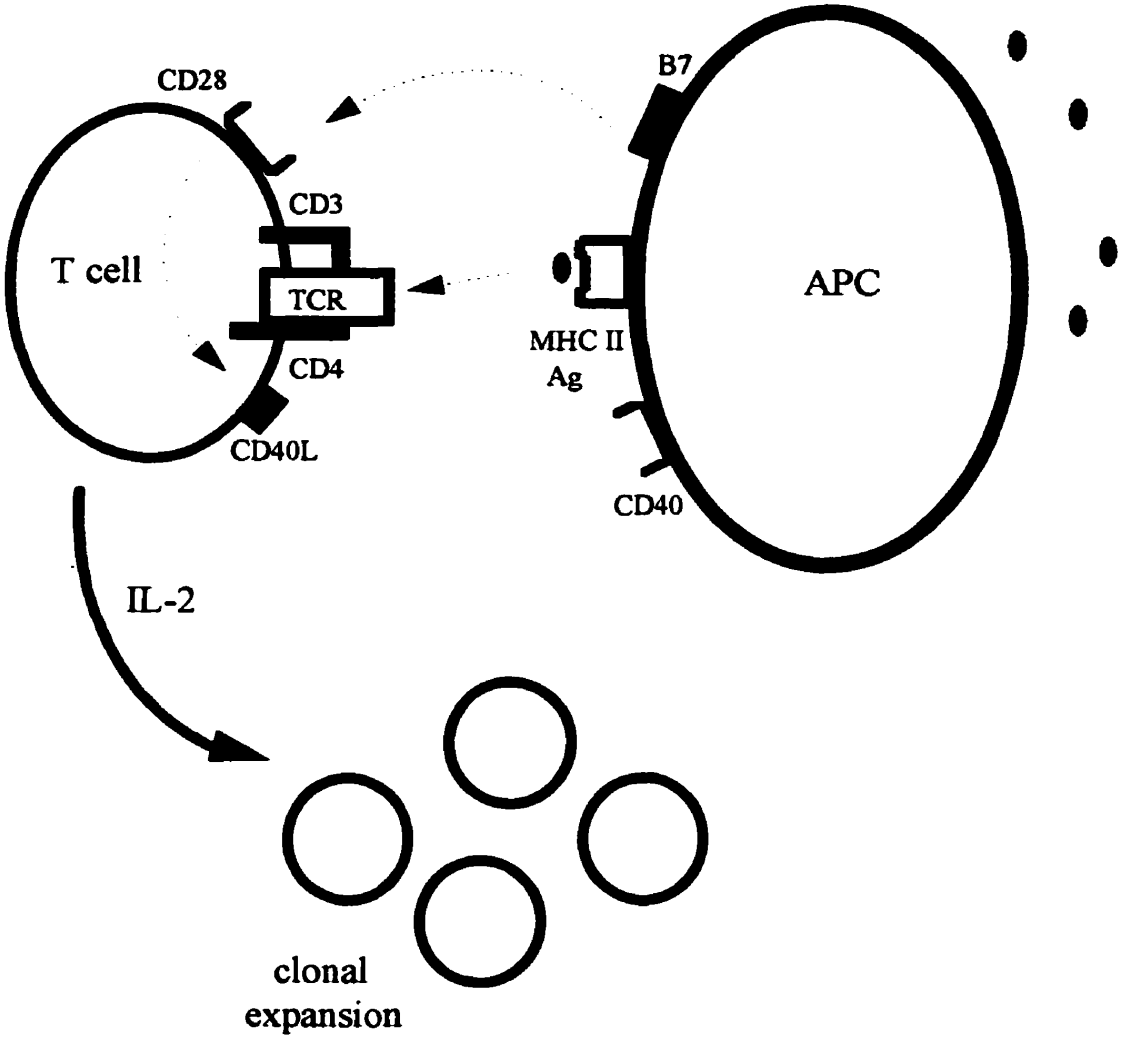


Diagram 1.3 Antigen presentation and co-stimulation

1.9c Germinal center formation

Subsequent to the initial antigen exposure, the B cell migrates into the B cell rich area called the follicle and commences formation of germinal centers [Gray et al, 1988]. These sites are oligoclonal [Kroese et al, 1987; Kuppers et al, 1993] and, on average, derived from the colonization of each follicle by 3 B cell blasts [Kroese et al, 1987; Liu et al, 1991]. Within the germinal center, B cells are considered to undergo positive selection [Liu etal, 1989; MacLennan et al, 1994], clonal expansion [Kroese et al, 1987; Jacob et al, 1991], somatic hypermutation [Berek et al, 1991; Jacob et al, 1991; 1992] and isotype switching [Feuillard et al, 1995; Liu et al, 1996]. A special component of this micro-evironment is the follicular dendritic cell (FDC), which takes up antigen and holds it on its surface as an immune complex in a native unprocessed form. B cells then take the antigen displayed by the FDC, process it and present it to T cells [MacLennan et al, 1994; Abbas, 1994f], inducing them to express CD40L and the necessary cytokines for isotype switching and Ig synthesis [Klaus et al, 1994; Diagram 1.4]. Finally the high affinity, isotype-switched B cells differentiate into memory B cells in the presence of prolonged CD40 signalling or plasma cells when CD40L is not present [Arpin et al, 1995].

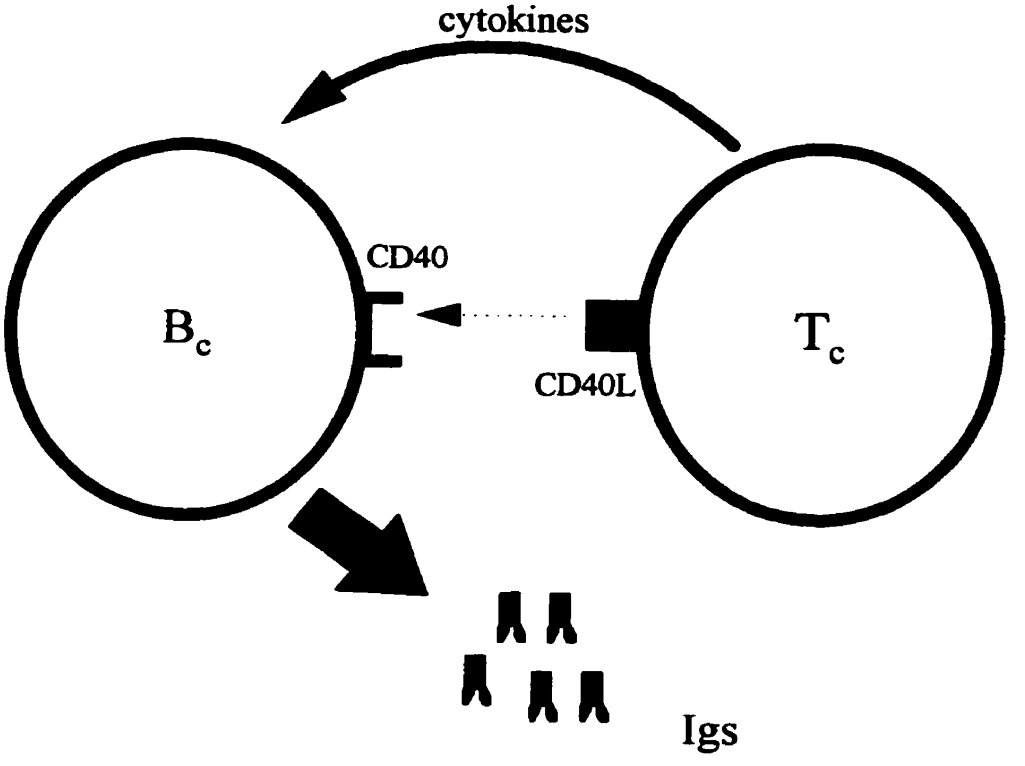


Diagram 1.4 T cell and B cell interaction

1.10 IgE

In 1921 it was observed that sensitivity to allergen could be transfer by a factor within the blood [Prausnitz et al, 1921; Sutton et al, 1997]. It was not until 1967, however, that this molecule was defined as an immunoglobulin and named IgE, due to the erythema that resulted from cutaneous allergen challenge of sensitized individuals [Ishizaka et al, 1967]. The IgE receptors are classified by affinity, FcεRI provides high affinity binding for IgE ($K_a = 10^{10}\ M^{-1}$), while FcεRII is of lower affinity ($K_a = 10^7\ M^{-1}$) [Sutton et al, 1997]. The FcεRI is expressed by mast cells [Metzger et al, 1992; Pawankar et al, 1997], dendritic cells [Maurer et al, 1996] eosinophils [Soussi Gounni et al, 1994] and basophils [Metzger et al, 1992; Sihra et al, 1997], while FcεRII is found on the surface of B cells [Jung et al, 1995], macrophages [Capron et al, 1986], Langerhans cells [Bieber et al, 1989], and also eosinophils [Capron et al, 1986]. In free form IgE has a half life of only a few days, however, when bound by FcεRs it is protected against degradation and can remain on the surface of inflammatory for months [Brostoff et al, 1996].

1.10a Mechanisms of action

IgE mediates the pathogenesis of allergy through a number of pathways. Firstly, antigen cross-linking of IgE-bound granulocytes results in the release of the pro-inflammatory mediators responsible for initiating the immediate type hypersensitivity response [Galli et al, 1993]. Furthermore, IgE amplifies this pathway by increasing the expression of FcεRI on mast cells [Yamaguchi et al, 1997] and by binding to CD23 on B cells, facilitating antigen presentation [Sutton et al, 1993]. Moreover, IgE may

regulate its own synthesis [Sherr et al, 1989], since low concentrations will allow cleavage of membrane CD23 (mCD23) into soluble fragments (sCD23) [Sarfati et al, 1988; Armant et al, 1995] that bind to CD21 on B cells and participate in positive feedback for IgE production [Armant et al, 1995]. As the concentration of IgE and IgE-antigen complexes rise, however, they will bind to both sCD23 and mCD23 and downregulate IgE synthesis [Gould et al, 1997].

1.10b IgE and allergy

Elevated levels of IgE within the sera of allergic individuals has been recognized as characteristic of allergy for over 30 years [Johansson et al, 1967]. In normal nonatopic individuals the serum concentration of total IgE is usually in the vicinity of 100 ng/ml, while in atopics this is generally over 900 ng/ml [Brostoff et al, 1996]. Antigen-specific IgE constitutes a high proportion (30-50%) of total IgE in allergic individuals following allergen exposure [Gleich et al, 1975]. Levels of both total and specific IgE appear to be influenced by factors such as age [Wittig et al, 1980], sex [Barbee et al, 1981], race [Grundbacher et al, 1985] and exposure to environmental hazards such as smoking [Burrows et al, 1981] and industrial pollution [Diaz-Sanchez et al, 1994a]. Men typically have higher levels of total serum IgE which, unlike women, does not decrease with age. However, the serum level of specific IgE is less in both men and women over age 55, compared to those aged 18-54 [Omenaas et al, 1994]. It also appears that gender may play a role in the level of serum IgE early life, since boys demonstrated a more rapid increase between birth and 2 years, while significant increases in girls were not observed until 2-4 years of age [Johnson et al, 1998].

In seasonal allergic rhinitics, allergen exposure is associated with significant increases in serum specific IgE that coincides with circulating pollen counts [Lichtenstein et al, 1973; Gleich et al, 1975; Naclerio et al, 1993]. Furthermore, the peripheral blood cells from seasonal allergic rhinitics have been shown to have a greater capacity for IgE production following IL-4 stimulation than non-atopic controls or from blood obtained out of season [Gagnon et al, 1993]. This appears to be a function of an increased number of IgE-synthesizing cells, since the amount of Ig produced per B cell was not altered among controls and hyper IgE patients [King et al, 1991]. Eighty percent of IgE+ cells within the blood of non-atopics, atopics and allergic are reportedly plasma cells [Donohoe et al, 1995].

Although IgA and IgG are also present within the nasal mucosa, their levels do not differ within nasal washings from allergic rhinitics compared to normal controls [Platts-Mills et al, 1976]. There are, however, high levels of total and allergen specific IgG_4, parallel to that observed of IgE [Merrett et al, 1976; Falconer et al, 1992; Aalberse et al, 1993; Panzani et al, 1996]. This is consistent with suggestions that IgG_4 and IgE are under similar regulatory control [Jeannin et al, 1998a]. IgG_4 is reported to have a much wider binding spectrum than IgE [Kobayashi et al, 1996]. Furthermore, since this IgG subclass does not fix complement and binds weakly to Fcγ receptors [Aalberse et al, 1983; van der Zee et al, 1986], therefore not initiating the allergic cascase, it has been termed a 'blocking antibody' [Prahl et al, 1981; Nakagawa et al, 1987; Mancini et al, 1991]. Allergic rhinitics receiving immunotherapy exhibit reduced serum total and specific IgE, while the amount of specific IgG_4 increases [Platts-Mills

et al, 1976; Nakagawa et al, 1987; Mancini et al, 1991]. Currently investigation is underway to determine mechanisms that induce the shift from B cell production of IgE to IgG_4 [Jeannin et al, 1998a].

1.11 Immunglobulin synthesis

Immunoglobulins (Ig) are 'Y' shaped molecules consisting of two distinct regions; the variable region, with two antigen binding sites on either arm; the constant region, which determines the Ig isotype and therefore the effector function of the antibody Immunoglobulins have both heavy and light chains. Generally, light chains have 1 variable and 1 constant domain, while heavy chains have 1 variable and 3 constant domains. IgM and IgE, however, have 4 heavy chain constant domains (Diagram 1.5). The nature of these regions is determined by two events of DNA deletion: *1)* VDJ rearrangement and *2)* class switch recombination.

1.11a VDJ rearrangement

All secondary immunoglobulins, *i.e.* other than IgM, produced by a B cell possess the same antigen specificity, determined by the amino acid sequence of the variable region. Within the germline, this region is encoded by three gene segments - a variable (V), diversity (D; not found in light chains) and joining (J) segment. During B cell development within the bone marrow, these segments are rearranged by the recognition of recombination signal sequences [Tonegawa et al, 1983], flanking the V, (D) and J segments, expression of recombination activating genes (RAG)1 and RAG2 [Schatz et al, 1989; van Gent et al, 1995]. This results in double-stranded DNA breaks and the alignment of V, (D) and J segmemts adjacent to one another [Schatz et al,

1989; McBlane et al, 1995; Diagram 1.6A,B]. After recombination, the double strand breaks are repaired through binding of protein kinases such as Ku80 [Mimori et al, 1986; Morozov et al, 1994; Jeggo et al, 1995]. Differential splicing of these three gene segments provides Ig molecules with a diversity of antigen binding sites.

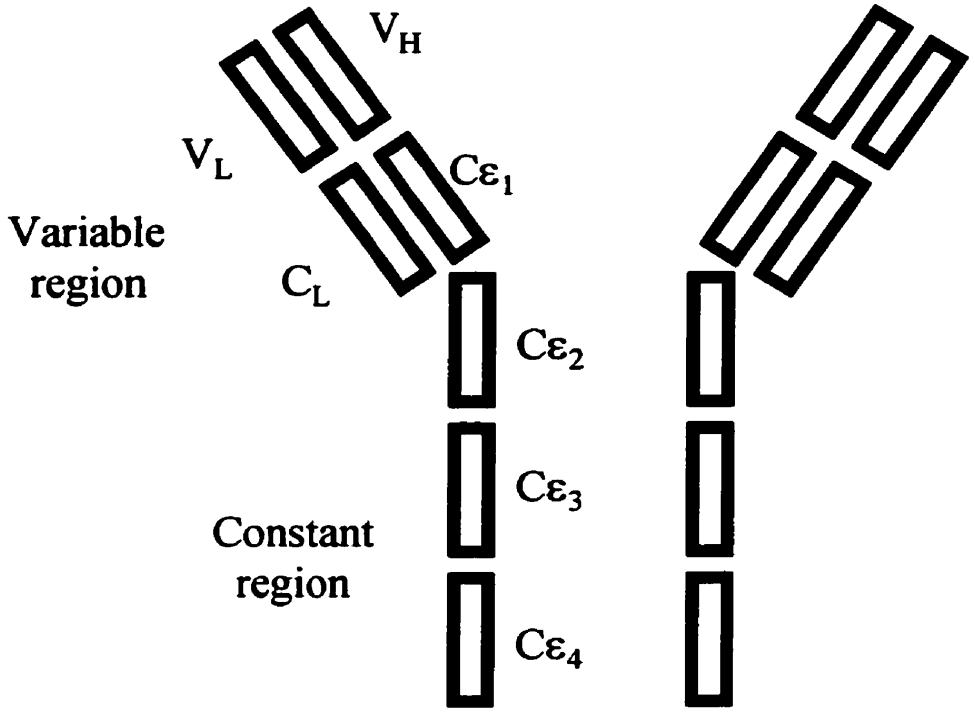


Diagram 1.5 The IgE molecule. Each immunglobulin has variable (V) and constant (C) regions.

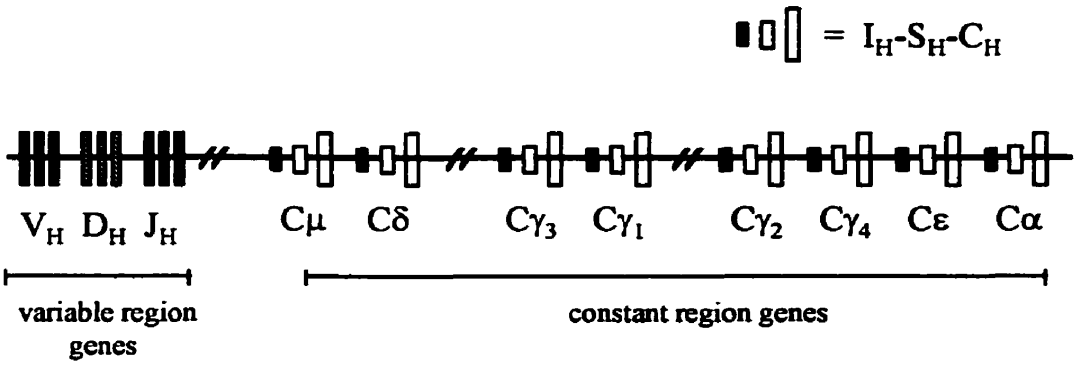
Further increases in antigen specificity take place within the germinal center by point mutation of V genes. This process is called affinity maturation or somatic mutation and transitions are frequently observed between G to A, A to G or C to T [Rajewsky et al, 1996]. Recently, RAG1 and RAG2 mRNA and double strand breaks at recombination signal sequences have been observed within germinal center B cells, suggesting that secondary VDJ rearrangements may occur at this site and contribute to the process of affinity maturation [Hikida et al, 1996; Han et al, 1997; Hikida et al, 1998].

1.11b Isotype switching

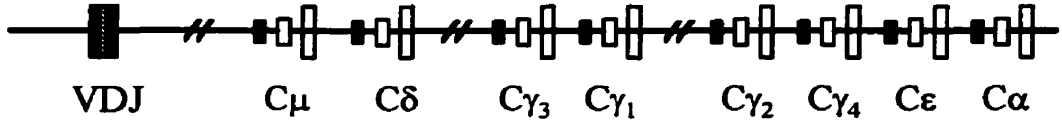
The effector function of an Ig is determined by the isotype, one of five constant regions (Ch), Cμ, Cδ, Cγ, Cε and Cα, which code for IgM, IgD, IgG, IgE and IgA, respectively (Diagram 1.6A). Mature mRNA transcripts, those that encode translatable protein, consist of a VDJ fragment spliced to one of these five Ch genes (VDJ-Ch), the Ch that is directly downstream. In virgin B cells, not previously exposed to antigen, Cμ lies in this position and as such these cells produce IgM [Snapper et al, 1990; Diagram 1.6C]. Within the germinal center, IgM+ B cells that have undergone somatic mutation are ready for isotype switching and will do so in response to further antigenic stimulation [Toellner et al, 1996; Liu et al, 1996]. Complete isotype switching requires 2 steps *1)* germline transcription and *2)* class switch recombination.

Class switch recombination occurs when highly repetitive DNA sequences called switch regions (Sh), located 5' to each Ch region [Shimizu et al, 1982], are rearranged and the intervening DNA is deleted [Iwasato et al, 1990; von Schwedler et al, 1990]. Since this is an irreversible event, and mistakes may compromise genome integrity, it is

Genomic DNA



B *VDJ rearrangment*



C *Transcription of Cμ gene*

VDJ-Cμ mRNA

Translation of Cμ mRNA

IgM

Diagram 1.6 Molecular events during B lymphocyte maturation.

tightly regulated. It is generally accepted that differential gene expression is, at least partially, due to DNA methylation patterns [Holliday et al, 1975; Bird et al, 1992]. Methyl groups are considered to either hide sequences required for transcription factor binding, recruit repressor proteins or induce structural changes in the chromatin itself [Razin et al, 1991; Singal et al, 1999]. In any case, it is recognized that *hypo*methylated genes are responsive to RNA polymerases and are most often transcriptionally active [Razin et al, 1980].

1.11b.i Germline transcription

Cass switch recombination relies on the increased accessibility, or hypomethylation, of specific Sh genes. The mechanism for increasing this accessibility is through germline gene transcription [Stavnezer et al, 1988]. Within the DNA, there is a promoter region (Ih) and switch region (Sh) lying 5' to each Ch (Diagram 1.7A). Germline transcription is initiated from this promoter site and runs through the Sh to just 3' of the Ch loci. Since switch region DNA is intronic, the Ih and Ch sequences are spliced together and form what is referred to as a germline or switch transcript (Ih-Ch) [Lennon et al, 1985; Lutzker et al, 1988]. Cytokines are responsible for targeting the Ih promoter sites, IL-4 and IL-13 have been shown to target the Iε gene [Gauchat et al, 1990; Ezernieks et al, 1996], while TGF-β appears to activate Iα [Briere et al, 1995]. In the Ch reading frame there are stop codons within the Ih exon, rendering these germline transcripts unable to code protein and they are therefore termed *sterile* [Lutzker et al, 1988]. This transcriptional event is considered to facilitate changes in the methylation pattern of Sh [Stavnezer-Nordgren et al, 1986; Burger et al, 1990] and

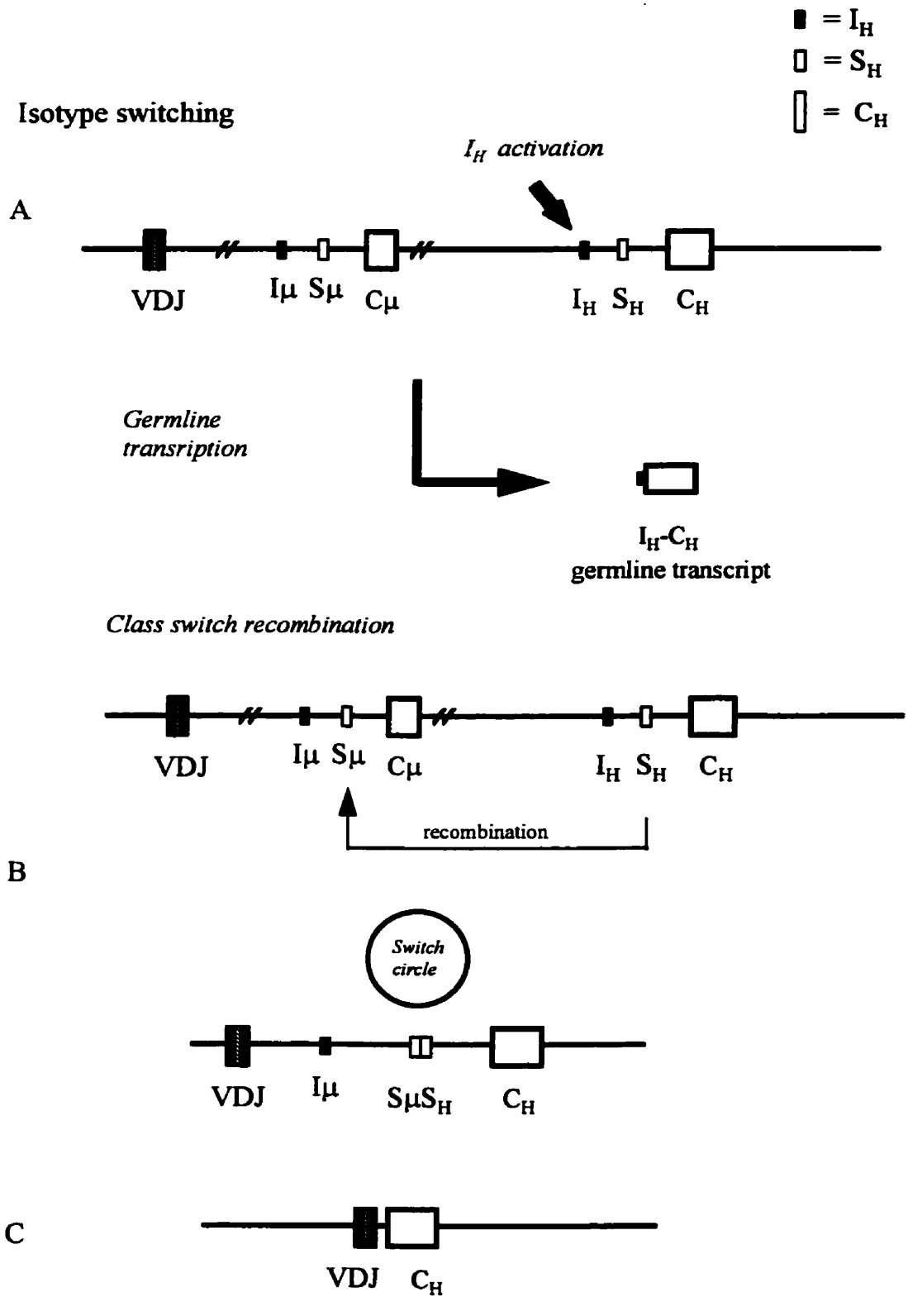


Diagram 1.7 Mechanism of isotype switching.

to uncover DnaseI hypersensitive sites [Schmitz et al, 1989; Berton et al, 1990; Snapper et al, 1997]. In addition, gene-targeted mutation of the Ih promoter has confirmed that germline transcription and/or transcripts play a direct role in class switch recombination [Jung et al, 1993; Zhang et al, 1993]. They have been suggested to direct recombination by forming triplex structures with the DNA at Sh regions [Collier et al, 1988; Reaban et al, 1994] or through transRNA splicing [Lutzker et al, 1988; Rothman et al, 1990]. Despite the present uncertainty pertaining to the exact function of germline transcripts, their production is considered a prerequisite for class switch recombination [Lorenz et al, 1995].

1.11b.ii Class switch recombination

Increased DNA hypersensitivity of the Sh regions has been shown [Stavnezer-Nordgren et al, 1986]. Furthermore like VDJ recombination, there are double strand breaks within the Sh regions, consistently located proximal to a 5'TGAG motif [Chou et al, 1993; Wuerffel et al, 1997]. Despite these findings, enzymes analogous to RAG1 and RAG2 are yet to be discovered.

The primary signal for class switch recombination is considered cross-linkage of the B cell surface molecule CD40 by its ligand glycoprotein 39 (CD40L) [Noelle et al, 1992a]. Disruption in the CD40L gene is associated with X-linked hyper-IgM and deficiency in Igs other than IgM and IgD [Aruffo et al, 1993; Allen et al, 1993; Korthauer et al, 1993]. CD40L is expressed by activated T cells [Noelle et al, 1992b], and its ligation with CD40 induces expression of B7 co-stimulatory molecules on B cells [Klaus et al, 1994]. Recombinase machinery induced by these co-stimulatory

pathways align the targeted Sh region so it is adjacent to Sμ. As these two sites come together, the intervening DNA forms a loop which is cleaved off, forming a switch circle [Iwasato et al, 1990; von Schwedler et al, 1990; Diagram 7B]. Knocking out the DNA repair protein Ku80 was seen to allow germline transcription, but not successful processing of mature Ig transcripts. This would indicate that, like VDJ recombination, repairing the double strand breaks is an important step in class switch recombination [Casellas et al, 1998]. In essence, this DNA rearrangement places the targeted Ch region adjacent to VDJ, forming the template by which RNA coding for this isotype is transcribed (Diagram 1.7C).

1.12 IgE synthesis

Our current understanding of the mechanisms that regulate IgE synthesis originate from a paper published in 1988. Del Prete and colleagues observed that anti-CD3 stimulated T cell clones and their supernatants could induce normal B cells to produce IgE. They went on to suggest that this was cytokine-associated, since a direct correlation with IL-4 and an inverse relationship with IFN-γ was observed [Del Prete et al, 1988]. This regulatory pathway was confirmed in the same year by Pene *et al.*, who demonstrated that addition of recombinant IL-4 (rIL-4) to normal B cell cultures induced IgE synthesis, while rIFN-γ inhibited this production [Pene et al, 1988b]. The importance of IL-4 was further evidenced by a failure to detect IgE in mice homozygous for a mutation that inactivated the IL-4 gene [Kuhn et al, 1991]. While IL-13 also induces IgE production, it appears to be 2-5 fold less potent than IL-4 [Punnonen et al, 1993; Zurawski et al, 1994].

1.12a Epsilon germline transcription

IL-4 and IL-13 induce the production of IgE by targeting the Iε promoter for germline transcription [Stavnezer et al, 1988; Rothman et al, 1990; Gauchat et al, 1990; Punnonen et al, 1995; Ezernieks et al, 1996]. The ε germline transcript was first cloned in 1990 by Gauchat and colleagues in B cells purified from peripheral blood mononuclear cells cultured with IL-4 and CD4+ T cells. It is 1.7 kb in length, consists of sequences coding for the Iε and Cε exons and is observed as early as 2 hours following stimulation. Mature, productive ε mRNA (VDJ-Cε) is 2.2 kb in length and is not seen in B cell cultures until 7 days following IL-4 stimulation, a time which coincides with IgE synthesis [Gauchat et al, 1990]. The ε promoter has response elements for a number of transcription factors including, STAT-6 [Fenghao et al, 1995; Ezernieks et al, 1996], NfκB [Delphin et al, 1995; Iciek et al, 1997] and B cell-specific activator protein (BSAP) [Wakatsuki et al, 1994; Thienes et al, 1997].

IL-4 and IL-13 both activate JAK1, while only IL-4 has been seen to induce JAK3 activity [Welham et al, 1995]. These proteins appear to be required for ε germline transcription, since protein kinase inhibitors arrest Sμ-Sε deletional switch recombination in IL-4/CD40 activated B cells [Loh et al, 1994; Yanagihara et al, 1995]. Activated Jak1/Jak3 proteins induce STAT-6 dimers to bind the DNA response element within the ε promoter and thereby activate germline transcription [Hou et al, 1994]. Furthermore, the critical role of this transcription factor has been conclusively proven using STAT-6 knock out mice, which were unresponsiveness to IL-4 and exhibited much lower levels of IgE [Shimoda et al, 1996; Takeda et al, 1996].

Similarly, mutating either the STAT-6 or NFκB response elements within this site or treating B cells with anti-sense BSAP oligonucleotides coincided with a reduction in ε germline promoter activity and Ig class switching in response to IL-4 [Wakatsuki et al, 1994; Delphin et al, 1995]. Since BSAP is the only known *B cell-specific* transcription factor with a response element within the ε germline promoter, Vercelli and colleagues suggested that activation of the BSAP site may regulate DNA interaction of STAT-6 and NFκB [Agresti et al, 1997]. Whether IL-13 also acts through NFκB and/or BSAP, independent of IL-4, remains to be determined.

Although both IL-4 and IL-13 mediate ε germline transcription, it appears that they do not do so to the same degree. IL-13 induces significantly lower levels of ε germline transcripts than IL-4 in immature human fetal B cells and can not induce pre-B cells to do so at all, indicating that this cytokine is not involved in pre-B cell differentiation [Punnonen et al, 1995]. Mediators that synergize with IL-4 to induce IgE synthesis include PGE_2 and TNF-α. Addition of PGE_2 to IL-4 and LPS treated murine splenic B cells induced germline ε transcripts and mature ε transcripts, more than IL-4 and LPS alone [Roper et al, 1995]. Furthermore, TNF-α enhances the IL-4-dependent expression of germline ε transcripts [Gauchat et al, 1992].

1.12b Sequential switching

IL-4 and IL-13 induce IgE as well as IgG_4 in human [Pene et al, 1988b; Punnonen et al, 1993; Zurawski et al, 1994] and IgG_1 in murine B cells [Berton et al, 1990]. The possibility that B cells may switch sequentially from IgG_4/IgG_1 to IgE came from observations that deleted DNA switch circles for IgE, that is fragments flanked by Sμ

and Sε, also contained Sγ sequences [Yoshida et al, 1990; Mills et al, 1992]. There is also a STAT-6 binding site within the γ_1 promoter of murine B cells [Delphin et al, 1995; Lundgren et al, 1994]. Factors that may reguate this sequential switch are currently being investigated. It appears that the dose of IL-4 may be important, since 10-30 times more was required to induce IgE rather than IgG_1 production in murine splenic B cells [Snapper et al, 1988]. IL-10 may also play a role in sequential switching, since it has been seen to reduce IL-4 and IL-13-induced expression of ε germline transcripts, while increasing their ability to promote production of γ_4 transcripts and IgG_4 synthesis in human B cells. However, IL-10 did not affect the production of IgE or IgG_4 in B cells that had not already undergone isotype switching, indicating that it is not a switch factor for IgG_4 [Jeannin et al, 1998a].

1.12c Class switch recombination to IgE

IL-4 and IL-13 are not sufficient for complete class switch recombination, production of mature ε mRNA and IgE protein. This was evidenced by the fact that culturing highly purified B cells with IL-4 or IL-13 only, failed to induce IgE synthesis. However, IgE was observed if activated T cells were added to the culture [Romagnani et al, 1990]. This indicated that B cell-T cell interactions may be important for IgE synthesis. Direct evidence to support this came from experiments using a double compartment system in which T and B cells were cultured in chambers separated by a microporous membrane permeable to molecules but not to cells. In this system, IL-4 did not induce IgE production from purified B cells, as was seen when T and B cells were co-cultured in the same comparment [Vercelli et al, 1989; Parronchi et al, 1990].

The importance of this contact has since been attributed to co-stimulatory molecules, such as CD40-CD40L [Noelle et al, 1992] and B7-CD28 [Valle et al, 1991; Linsley et al, 1991; Borriello et al, 1997]. The necessity of these molecules is demonstrated in knockout experiments, whereby CD40 -/-, CD40L -/- and B7 -/- mice were unable to produce secondary Ig (IgG, IgE or IgA) or form germinal centers [Kawabe et al, 1994; Xu et al, 1994; Borriello et al, 1997]. Furthermore, these pathways are positively linked, since CD40 activation upregulates expression B7 [Roy et al, 1995; Ranheim et al, 1995] and CD28 ligation enhances expression of CD40L [Klaus et al, 1994]. Activated CD40 signals through both NFκB [Lalmanach-Girard et al, 1993; Berberich et al, 1994; Iciek et al, 1997] and BSAP [Thienes et al, 1997]. However, mutation of the STAT-6 binding site within the ε promoter did not arrest CD40-induced ε germline transcription, indicating that its action is independent of this transcription factor pathway [Warren et al, 1995]. Other ligand-receptor pairs, such as interaction between CD2 on T cells and CD58 on B cells, have also been reported to provide co-stimulatory help required for IL-4 stimulated B cells to produce mature ε RNA transcripts [Diaz-Sanchez et al, 1994b].

1.13 Local IgE

The symptoms and characteristics of allergic disease are a consequence, either direct or indirect, of the IgE-mediated inflammatory cascade. A diagnosis of allergic rhinitis, as with most allergic diseases, consists of the manifestation of nasal symptoms, a positive skin test to allergen and increased serum levels of specific IgE. While most allergic rhinitics are positive on all three points, some present with clinical symptoms

but appear non-allergic upon testing. One group investigated patients with apparent symptoms of allergic rhinitis but negative skin reactions to specific allergen and no or undetectable levels of specific serum IgE. Nasal provocation confirmed allergen sensitivity in these patients and also resulted in an increase in specific IgE in nasal secretions, amounts comparable to allergic rhinitics with positive skin tests and raised serum specific IgE [Huggins et al, 1975]. The presence of IgE within the tissue may be due to plasma exudation of free IgE, infiltrating IgE-bound inflammatory cells or migrating IgE-producing plasma cells, since B cells isolated from nasal lavage following allergen challenge were producing IgE protein and epsilon mRNA [Diaz-Sanchez et al, 1994a; Zurcher et al, 1996; Diaz-Sanchez et al, 1997]. Yet a further possibility is the local commitment of B cells to IgE production, that is isotype switching, within the nasal mucosa itself.

A first point of evidence in support of local commitment to IgE is the fact that there is a resident population of B cells within the nasal mucosa [Winther et al, 1987; Davidsson et al, 1994]. These B cells have been characterized as CD19+/CD20+ and may also express CD23, since this marker has been detected within the nasal mucosa of patients with allergic rhinitis [Davidsson et al, 1994]. B cell expression of CD23 is reportedly lost after isotype switching and therefore its presence on a B cell identifies it as a pre-switched cell [Kikutani et al, 1986; Pascual et al, 1994]. These B lymphocytes may derive from a subset of IgM+IgD+CD27- cells, observed to make up a large component of peripheral blood B cells [Klein et al, 1997; Klein et al, 1998]. Although the presence of these cells within the nasal mucosa has not been determined,

IgM+ B lymphocytes have been observed within nasal secretions and tissue, significantly more in allergics compared to controls [Illum et al, 1978].

Secondly, the factors required to induce B cells to isotype switch to IgE appear to be expressed by cells within the nasal tissue. There are T cells expressing IL-4 and IL-13 mRNA within this tissue [Ying et al, 1994; Ghaffar et al, 1997] and the number of activated T cells (CD25+) are also increased [Hellquist et al, 1992], indicating that they are expressing CD40L [Klaus et al, 1994]. It is also conceivable that other cells, in addition to the T cell, may provide the necessary B cell help.

Mast cells and eosinophils are characterized by constitutive expression of CD40L [Gauchat et al, 1993; Banchereau et al, 1994; Gauchat et al, 1995]. Within the nasal mucosa of sensitized individuals, these cells are present in ample number and are bound to IgE in anticipation of allergen exposure [Soussi Gounni et al, 1994; Pawankar et al, 1997]. In this event, allergen cross-linkage induces these granulocytes, particularly the mast cells, to rapidly release IL-4 [Bradding et al, 1992; Wang et al, 1999] and commence production of IL-13 [Pawankar et al, 1995]. Furthermore, since T cells must rely on antigen presention for activation, induction of CD40L and Th2 cytokine expression, it is conceivable that the granulocytes may perform this function with superior efficiency. Within the tissue, mast cells have been considered mainly to associate with T cells, providing the primary source of IL-4 for Th2 phenotype development [Wang et al, 1999]. They have, however, been observed in direct contact with B cells within tonsillar tissue [Ganzer et al, 1988], suggesting their ability to do so within the nasal mucosa. As such, it appears that the factors required to induce a B

cell to isotype switch in favor of IgE are in fact present within the nasal mucosa of individuals with allergic rhinitis.

1.14 Treatment of allergic rhinitis

1.14a Anti-histamines and nasal decongestants

There are a number of treatments currently in use for the management of allergic rhinitis. Many of them are aimed at circumventing the symptoms that arise from neural activation and effects of vessel dilation and glandular activity. Anti-histamines block the effects of histamine on H1 receptors, thereby reducing the characteristic sneezing, itching and rhinorrhea [Simons et al, 1994]. Nasal decongestants produce vasoconstriction of capacitance vessels and subsequently reduce mucosal swelling [Dushay et al, 1989]. While these agents are used by almost all patients with allergic rhinitis, at some time or other, they mainly treat the symptoms which are subsequent to the allergic response, rather than making an attempt to inhibit the inflammatory process *per se*. As such, they are commonly referred to as 'rescue medication'.

1.14b Immunotherapy

Immunotherapy has been recognized as an effective treatment for allergy since 1911 [Noon et al, 1911]. It involves injection of increasing doses of the allergen over a prolonged period of time, in effect giving the body a chance to develop a protective rather than a hypersensitive response. While this treatment has proven highly effective in some patients, it is not successful in all cases. The mechanisms underlying the efficacy of this treatment are not completely understood, however, it appears to induce a Th1 cytokine expression [Hamid et al, 1997a] and IgG production [McHugh et al, 1990]. Indeed, the IgG_4 subclass is significantly increased in patients

that have received immunotherapy and is correlated with improved clinical symptoms [Ohashi et al, 1997]. As such, it is referred to as a 'blocking antibody' and it's production during immunotherapy may provide the necessary break in the positive feedback mechanisms of IgE synthesis. There are a number of factors which make immunotherapy a less attractive choice of treatment. It is not successful for all allergen sensitivities, it requires injections over a period of 3-5 years and the symptom relief is gradual as it takes time to shift the individual's immune response.

1.14c Glucocorticosteroids

Glucocorticosteroids are well recognized to have potent anti-inflammatory effects and are generally considered the number one choice of treatment for allergic disease. They act at a number of levels, inhibiting the production of inflammatory mediators such as cytokines, chemokines and adhesion molecules important for leukocyte homing [Schleimer et al, 1997].

1.14c.i Mechanisms of action

Glucocorticoids (GC) mediate their anti-inflammatory activity through specific cytoplasmic receptors (GCR) [Barnes et al, 1997]. A number of mechanisms are involved, including the binding of GC:GCR complexes to negative response elements within the promoter of the target gene [Drouin et al, 1993] and interference with transcription factors, most notably NFκB [Barnes et al, 1993]. NFκB mediates gene transcription initiated by a number of cytokine and signaling events, including CD40 activation [Lalmanach-Girard et al, 1993; Berberich et al, 1994; Iciek et al, 1997]. This transcription factor exists within the cytoplasm complexed to the inhibitory

protein IκB, which prevents it from entering the nuclei [Baldwin et al, 1996]. Under normal circumstances, activation of receptors that signal through NFκB causes phosphorylation of IκB, which rapidly degrade and leave NFκB free to translocation to the nucleus and bind DNA [Chen et al, 1996]. However, the GC-GCR complex induces the expression of IκB, which can bind to NFκB and cause its dissociation from its response element and subsequent migration back to the cytoplasm [Scheinman et al, 1995; Barnes et al, 1997].

1.14c.ii Inhaled coriticosteroids

Inhaled corticosteroids are highly lipophillic, which allows for rapid entry of the steroid molecule into the respiratory mucosa. Most inhaled steroids are used in their pharmacologically active form, intended to provide localized therapy with immediate drug activity at the site of delivery. Furthermore, these preparations have relatively low bioavailability and rapid clearance after absorption, which minimizes the systemic side effects [Mackie et al, 1996].

1.14c.iii Fluticasone propionate

Fluticasone propionate (FP) is a topical corticosteroid developed using cortisol as the basic structure. Addition of the fluoromethyl analogue at position 17, however, has greatly improved the efficacy of the molecule [Phillipps et al, 1990]. FP is 300 times more lipophillic than budesonide and 3 times more than beclomethasone dipropionate [Johnson et al, 1996]. The complex of FP and the glucocorticoid receptor binds to its DNA response element on target genes or interacts with AP-1 and NFκB at significantly lower concentrations than either dexamethasone or budesonide [Adcock

et al, 1996; Johnson et al, 1998]. In human lung tissue, FP has the fastest uptake and retention [Hogger et al, 1993] and the greatest stability of the steroid receptor complex, compared to other topical nasal steroids [Barnes et al, 1993]. Furthemore, the half-life of FP is more than 10 hours, longer than other topical steroids [Johnson et al, 1996].

In patients with allergic rhinitis, pre-treatment with FP inhibits the onset of clinical symptoms induced by acute antigen challenge [Rak et al, 1994]. Furthermore, it is associated with a reduction in the number of inflammatory cells, mast cells, eosinophils, CD4+ T cells and the pro-allergic cytokines, IL-4, IL-5 and IL-13 [Rak et al, 1994; Ying et al, 1994; Ghaffar et al, 1997; Masuyama et al, 1998].

1.15 Future treatment for allergic rhinitis

In light of the possibility that there may indeed be local synthesis of IgE within allergic nasal mucosa, the success of intra-nasal steroid administration becomes clear. Furthermore, it indicates that treatments currently considered of inferior efficiency for reducing the symptoms of nasal allergy may merely be due to lack of a local administration regime. Future therapies may include not only interfering with signalling pathways necessary for isotype switching to IgE in the nasal mucosa, such as IL-4, IL-13 or CD40L, but also the emphasis on the local administration of these reagents.

1.16 Rationale

Although the provenance of IgE within respiratory tissue has primarily been attributed to its production within regional lymphoid organs, there are B cells within the nasal mucosa. Furthermore, the two signals required to induce a B cell to undergo isotype switching to IgE; *i.e.* the presence of cells that express *a)* CD40L and *b)* the IgE-switch factors IL-4 and IL-13, are found within nasal mucosal tissue obtained from individuals with allergic rhinitis.

1.17 Hypothesis

In light of this, the present work investigates whether these B cells residing within the nasal mucosa undergo *local* ε germline transcription, the initial and requisite step for class switch recombination to IgE, in response to allergen exposure. Owing to the potent effects of corticosteroids on the clinical symptoms of allergic rhinitis and their ability to reduce IL-4 and IL-13, the effect of fluticasone propionate pretreatment on ε germline transcription was also investigated.

1.18 Aims

1.18a General Aims

The general aim of this thesis was to examine whether B cells within the nasal mucosa of individuals with allergic rhinitis undergo *local* ε germline transcription following allergen exposure and if this event could be inhibited by pre-treating the patients with topical corticosteroid.

1.18b Specific Aims

The specific aims of this work were:

1. To determine if *in vivo* allergen challenge of asymptomatic seasonal allergic rhinitis patients would induce an increase in the number of cells expressing mature ε RNA, ε germline transcripts and mRNA coding for IL-4. To ascertain whether a 6 week pre-treatment with topical fluticasone propionate could inhibit these increases.

2. To determine whether *natural* exposure of seasonal allergic rhinitis patients to allergen would also induce an increase in the number of cells expressing mature ε RNA, ε germline transcripts, mRNA coding for IL-4 and whether a 6 week pre-treatment with topical fluticasone propionate would inhibit these changes.

3. To determine whether *ex vivo* allergen challenge, using expanted nasal mucosal tissue, from asymptomatic seasonal allergic rhinitis patients would also exhibit an increase in the expression of mature ε mRNA, germline ε transcripts and mRNA coding for IL-4 and IL-13.

Chapter 2

2.0 MATERIALS AND METHODS

To address the questions put forward in this thesis, three major studies were performed. Initially, the response to allergen within the nasal mucosa of individuals with seasonal allergic rhinitis was assessed using a model of the disease, generated by acute nasal provocation with specific allergen. This model has been used previously to study the immediate type hypersensitive response of the nose [Varney et al, 1992; Durham et al, 1992; Rak et al, 1994]. A second study was performed to confirm that changes observed in response to the acute challenge were representative of naturally occurring disease. To do this a similar protocol was employed, differing only by the fact that exposure to allergen was natural that is, during the pollen season. These two studies assessed effects of *in vivo* exposure to allergen and therefore the possibility that these observed changes were due to cell infiltration, rather than a local tissue response, still remained. For this reason, nasal tissue was cultured with specific allergen. Using this nasal mucosal explant technique, the possibility of systemic infiltration was eliminated and the changes observed following culture with allergen were assured to be derived solely from tissue constituents.

2.1 Patients

All patients recruited had a history of seasonal allergic rhinitis (SAR) for at least 2 years with a positive skin test of greater than 5 mm for Timothy grass (*Phleum*

pratense) or ragweed (a mix of giant and short; *Ambrosia trifida* and *artemisiifolia*) pollen extract. They were all non-smokers and had not received topical or oral steroids in the previous 6 months or immuntherapy in the last 5 years. Normal subjects and non-atopic controls had no nasal symptoms, were negative on skin testing and were also non-smokers. Twenty-one patients for the allergen challenge study were recruited at the Royal Brompton Hospital, London, UK, thirty-three patients were recruited for the natural exposure study at the Allergy Clinic at the Shalgrenska Hospital, Gotenburg, Sweden, while thirteen allergic patients and nine non-allergic controls were obtained for *ex vivo* allergen exposure at the Jewish General Hospital and the Nôtre Dame Hospital, Montréal, Canada. All protocols were assessed and granted approval by the Ethics Committee of the respective institutions and patients were informed of the procedures before they were asked to give their written consent.

2.2 Study design

2.2a In vivo allergen challenge

Patients were randomized to receive a 6-week pre-treament with topical nasal coricosteroid (200 μg fluticasone propionate [FP], two sprays per day) or a matched placebo containing the aqueous diluent. After treatment, all patients underwent local nasal provocation with grass pollen extract. This was performed by applying a 4 mm filter paper disc that had absorbed 1000 biological units of grass pollen extract to the under-surface of the inferior nasal turbinate for a period of 10 minutes (Plate 2.1a,b). Pre- and post-challenge biopsies were obtained using a 2.5 mm diameter cup-and-ring forceps, the Gerritsma forceps [Fokkens et al, 1988; Durham et al, 1992; Plate 2.2].

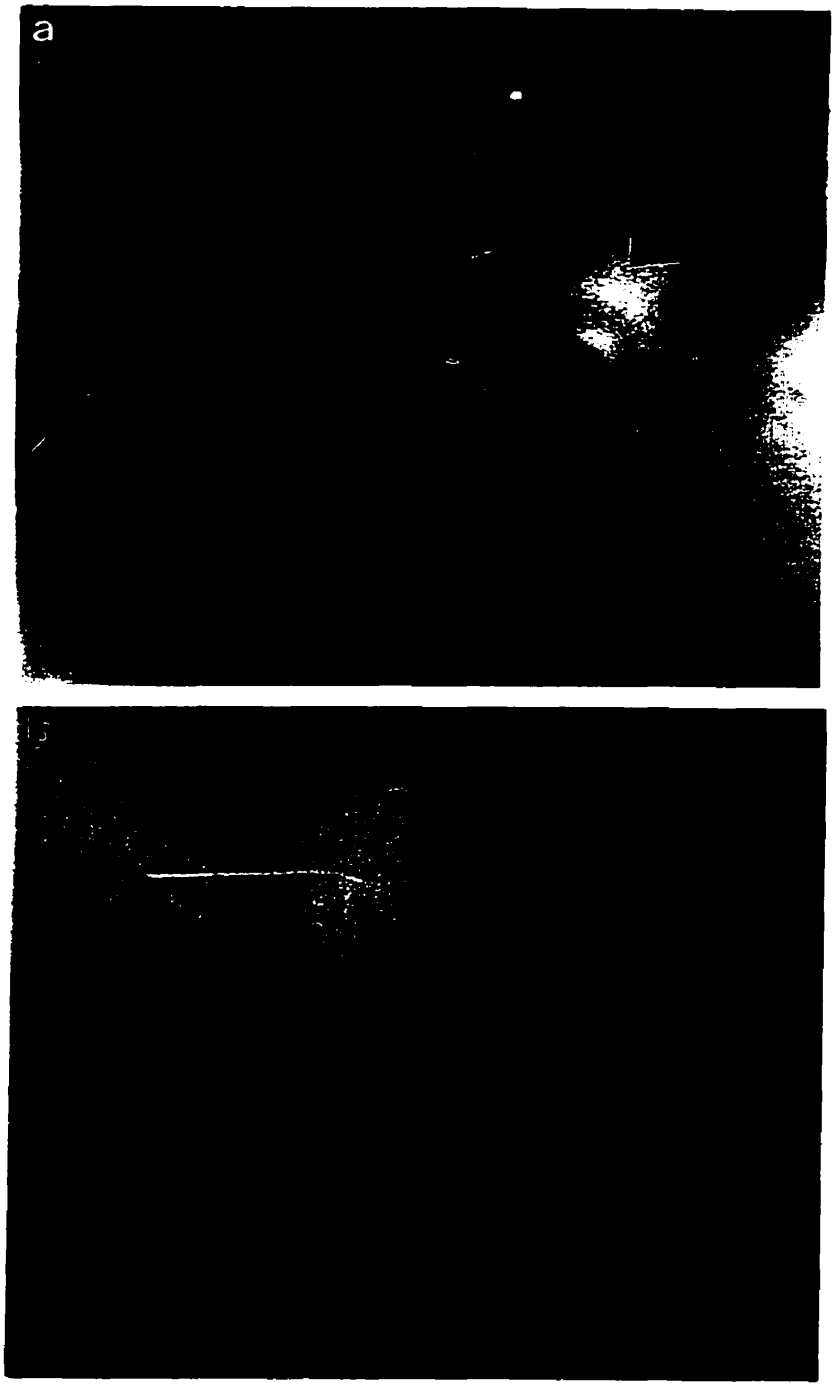


Plate 2.1 Intra-nasal allergen challenge performed by applying a 4 mm filter paper disc soaked in allergen extract to the undersurface of the inferior turbinate (a). A string was attached to the disc and taped to the subjects' cheek for easy removal (b).

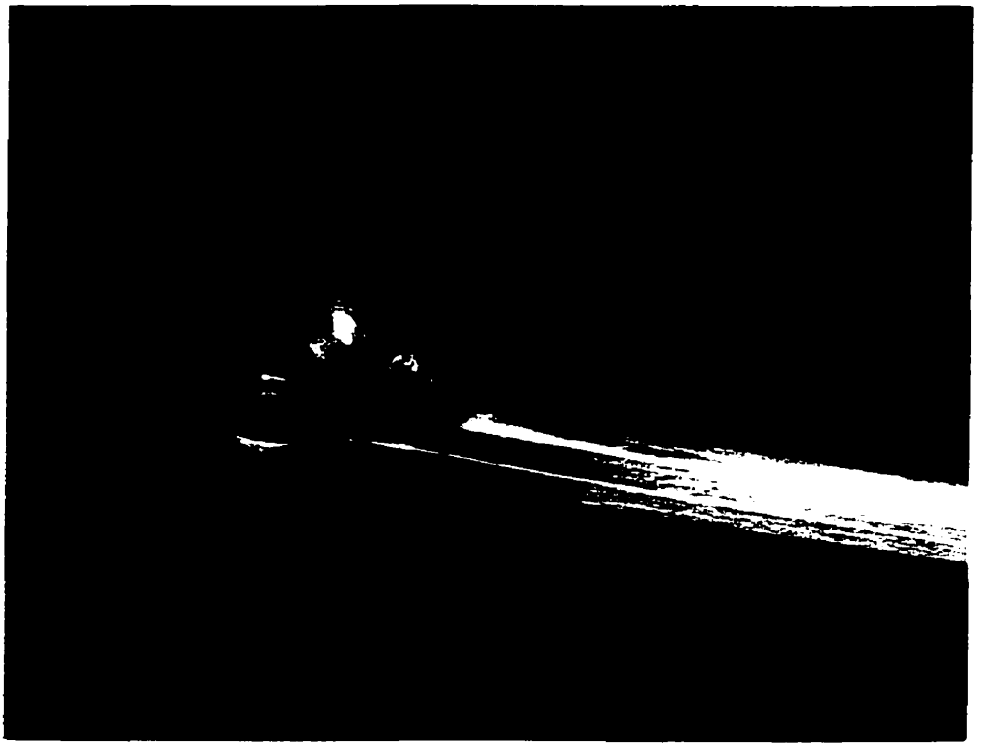


Plate 2.2 The Gerritsma forceps. This instrument has been developed especially for the purpose of obtaining high quality nasal biopsies. The cup-and-ring feature ensures that the tissue is not crushed and a large portion of the epithelium remains intact.

Patients were asked to record the number of sneezes they experienced between *1)* 0 and 60 min and *2)* 1 and 24 hours following allergen challenge. Weight of nasal secretions was ascertained by weighing all tissues used by the patient over the 24 hour period. The degree of nostril blockage was assessed by a visual analogue score, a rating on a scale of 1 - 10, in answer to the question *"How blocked does your nose feel?"*. These procedures were performed both within one hour and again 24 hours following allergen challenge.

2.2b Natural allergen exposure

Patients were recruited outside the grass pollen season and randomized to receive either a six week course of FP aqueous nasal spray (200μg) twice daily or a matched placebo control. In effect, this study was identical to that described for *in vivo* allergen challenge, however, patients were exposed natually to allergen during the pollen season. Clinical symptoms were assessed by obtaining weekly visual analogue scores (VAS) to indicate the patients' overall nasal symptoms. This was done by asking the patient to place a mark on a line of 10 cm in length in response to the question *"How has your hayfever been this week?"*. Measuring the distance from 0 cm to this mark indicated the degree of symptom improvement [Varney et al, 1991]. Biopsies from the inferior turbinate were obtained using the Gerritsma forceps, both before and during the season.

2.2c Ex vivo allergen exposure

For the culture of human nasal mucosal tissue, freshly prepared bicarbonate-buffered culture medium (BCM) was employed in all experiments. This was made by dissolving mineral essential medium (MEM) powder with Earl's salts, L-glutamine and sodium

bicarbonate in ultra-purified distilled water. This solution was supplemented with 20 ml/litre of MEM amino acid solution 50X, 10 ml/litre of sodium pyruvate, 10 ml/litre of vitamin solution, 0.1μg/ml of bovine insulin, 0.1μg/ml vitamin A, 0.1 μg/ml of hydrocortisone and 50 μg/ml of gentamycin, adjusted to a pH of 7.25 and filter sterilized, leaving it at a final pH of 7.35. This is a defined medium previously employed for culture of animal [Placke et al, 1987; Dandurand et al, 1994] and human tissue [Minshall et al, 1997c].

Mucosal tissue from the inferior turbinate was obtained from patients undergoing sinus surgery using a biting forceps or by surgical resection. Tissue was obtained directly from the operating room (OR) and placed immediately in BCM for transport to the laboratory (Plate 2.3). Under sterile conditions, tissue was serially sectioned ensuring that each piece consisted of both an epithelial and submuocsal layer. Although the size of the section varied depending on the quantity of resected tissue, the average length of basement membrane was 0.5 cm. In some protocols, the tissue is placed directly in the culture well, allowed to adhere to the plastic, and then the medium is added. Since the epithelium of the nasal mucosa is exposed to the atmosphere normally, *in vivo*, these experiments were carried out by placing the tissue on well inserts, consisting of a 0.4μm filter, that allowed it to be bathed but not immersed in medium. With this technique the tissue retains the epithelial/air interface. Tissue was cultured with either 250 μl of ragweed allergen (*Ambrosia trifida* and *artemisiifolia*; GS) of increasing doses (5-1000 PNU/ml) or in medium alone and kept in 5% CO_2/95% air for a period of 24 hours (Diagram 2.1).

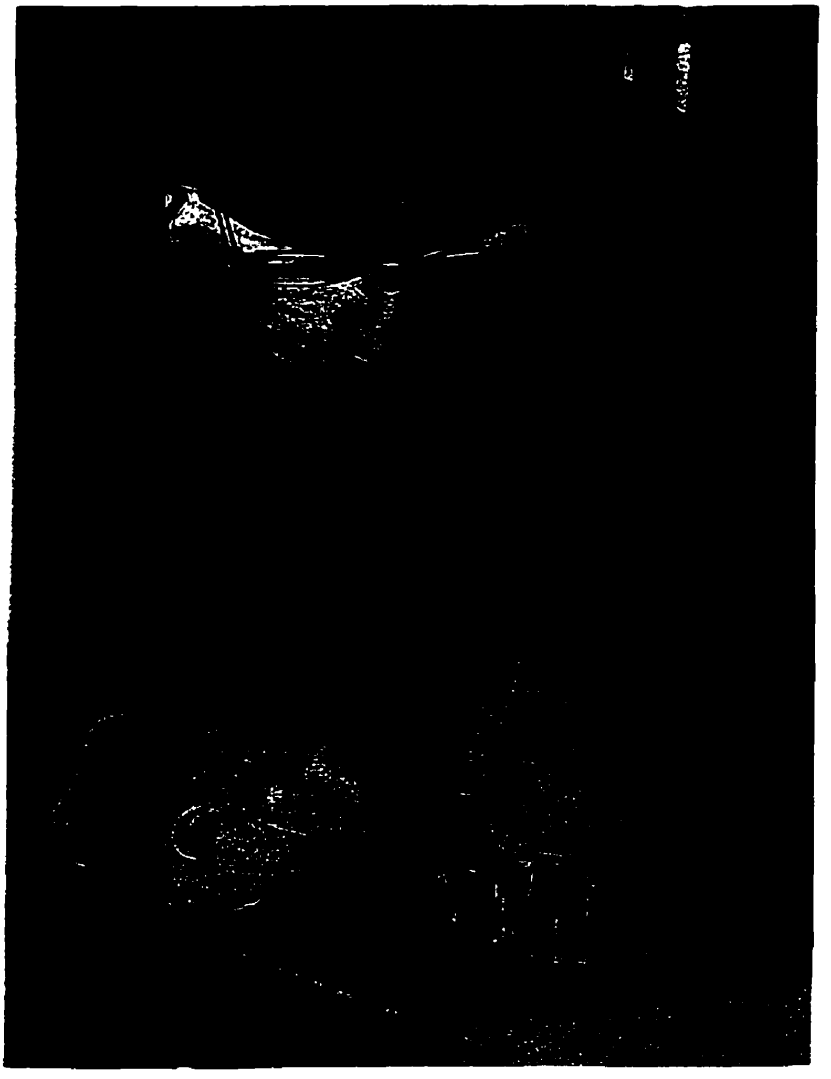


Plate 2.3 Nasal tissue for explant experiments was obtained directly from the OR and placed immediately in culture medium for transport to the laboratory.

2.3 Tissue processing

For this study, tissue was examined for the expression of various proteins and mRNA products. This was achieved using the techniques of immunocytochemistry and *in situ* hybridization, respectively. Co-localization studies were performed by combining one or more of these techniques. As such, the tissue obtained for study was processed according to standard protocols for immunocytochemistry and *in situ* hybridization [Hamid et al, 1987; Frew et al, 1988].

2.3a Fixation

Inferior turbinate biopsies processed for *in situ* hybridization were fixed prior to freezing for maximum retention of RNA. The importance of this step has been demonstrated in experiments with unfixed tissue, that exhibited not only a disrupted morphology but also loss of the mRNA [McCabe et al, 1986]. Aldehyde fixation is the most common for RNA hybridization. While glutaraldehyde and buffered formalin are excellent fixatives for morphological analysis, comparative studies indicate the particular suitability of paraformaldehyde for cell and cryostat preparations [Gee et al, 1983; Lawrence et al, 1985]. This fixative preserves the tissue in a morphologically intact state while providing maximum accessibility to the mRNA within the cell. In this thesis, a 4% paraformaldehyde (PF) solution was employed by dissolving PF powder in 0.1M phosphate buffered saline (PBS) at a temperature between 55-60°C. This solution was not allowed to rise above 60°C, as the PF will denature. Biopsies were placed in this solution at room termperature for 2 hours and then washed three times in 15% sucrose/PBS for one hour each, with the

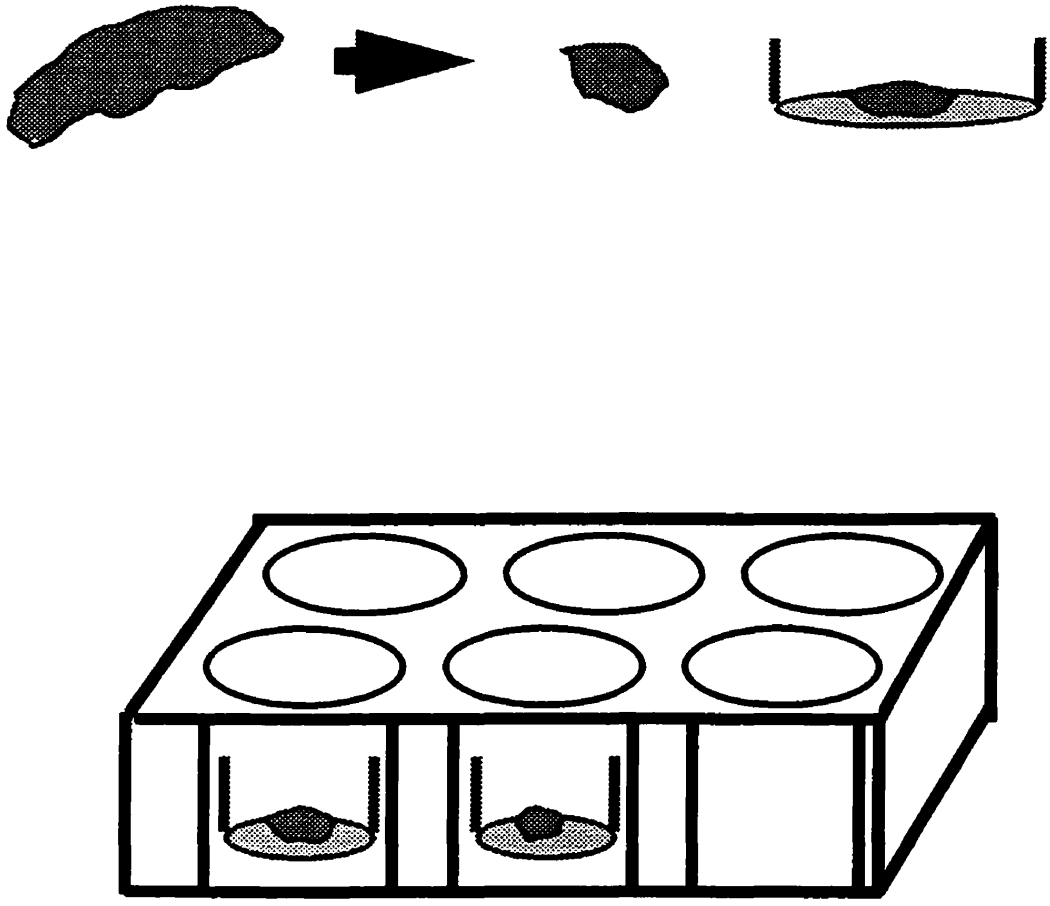


Diagram 2.1 Schematic representation of the nasal mucosal explant system

last wash left overnight. The importance of these washes is two-fold; *1)* to thoroughly rinse the tissue of PF and *2)* the hypertonicity of the solution draws the excess water out of the tissue, which is important because if omitted the tissue will crack as the freezing water expands. The time is also important, since over-fixation will decrease hybridization signal by masking the mRNA.

For immunocytochemistry of cell surface antigens the best results are obtained when tissue is freshly frozen. For these experiments, upon receiving the tissue it was placed immediately in PBS and frozen within 30 minutes. Cell surface antigens are highly susceptible to routine fixation, which permeabilize membranes. As such, in these experiments tissue was subjected to a short, mild fixation in an acetone/methnanol (60:40) solution.

2.3b Blocking and Sectioning

There are a number of embedding mediums employed for blocking tissue, all of which have the shared feature of being a liquid in one phase and a solid at another. Paraffin, resins and glycol methacrylate can be used for sectioning of semi- and ultra-thin sections, however, they are not always suitable for the detection of more labile surface antigens and mRNA. In this study, all tissue was fresh frozen and blocked using the viscous embedding medium OCT (optimal cutting temperature). This was done by inserting the tissue in a small droplet of the OCT and immersing it in isopentane, cooled with liquid nitrogen. Almost immediately the OCT will freeze to a solid, at which point it is referred to as a 'tissue block'. When blocking nasal mucosal biopsies it is important to orientate the tissue in cross-section, so that each section of

the block consists of both the epithelial and submucosal layer. Once frozen, blocks are stored at -80°C until use.

For *in situ* hybridization, blocks were cryostat sectioned at a thickness of 8-10 microns. Owing to the somewhat rigorous nature of this technique, with its multiple washes and enzymatic treatments, it is important to section tissue onto glass slides coated with a poly-L-lysine (PLL) solution. Poly-L-lysine gives the slides a positive charge and provides firm adhesion of the tissue [Liesi et al, 1986]. Furthermore, slides for ISH are incubated overnight at 37°C to allow dehydration of the tissue and maximum adherence. Slides are stored at -80°C until use.

For immunocytochemistry, crytostat sections of 5-8 microns were cut and slides were fixed in an acetone/methanol (60:40) solution for no longer than 7 minutes. PLL coated slides are not required for this technique, partly due to the fact that fixation is performed post-sectioning but also because immunostaining does not involve ezymatic treatment, as *in situ* hybrdization does. Fixation in acetone and methanol dehydrates the slides and as such they do not need to be incubated overnight. Slides for ICC are wrapped in aluminum foil and stored at -20°C.

2.4 Basic principles of molecular biology

Until relatively recently, our understanding of the molecular mechanisms of the cell were rather limited. While the discovery of nucleic acid was well over 150 years ago, it has only been in the last sixty years or so that discoveries in cellular biology have given rise to a field of investigation termed *molecular* biology. Many techniques have been developed to study and learn more about the inner workings of cells as well as subcellular alterations characterisitic of disease pathology. This thesis examined the expression, or lack thereof, certain mRNA and protein products using *in situ* hybridization (ISH) and immunocytochemistry (ICC). These techniques involve the generation of polynucleotide fragments complementary to endogenously produced nucleic acid sequences and the detection of proteins using specific antibodies, respectively. The following section provides an overview of the discoveries leading to our current understanding of DNA and the processes by which a cell produces protein.

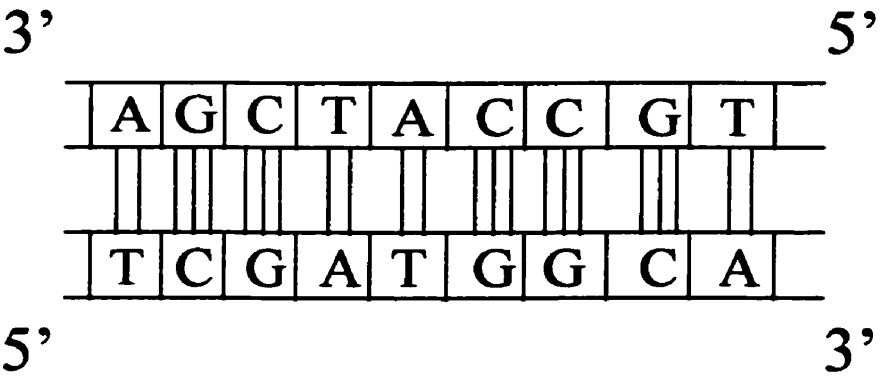
2.4a Nucleic acid

In 1868 Miescher was the first to isolate the phosphorous rich compound he called nuclein from the nuclei of leukocytes [Mirsky et al, 1968; Southin 1991a]. This compound was subsequently named *nucleic acid* and found to be composed of 4 nitrogeous bases: adenine (A) and guanine (G) (the purines) and cytosine (C), thymine (T) and uracil (U; the pyrimidines). In 1944, the classic experiments of Avery, MacLeod and McCarty demonstrated that genetic information is stored as nucleic

acid, not protien as was previously believed [Avery et al, 1944; Southin, 1991b]. There are two types of nucleic acid, distinguished by the pentose bound to the nitrogenous base. Deoxyribonucleic acid (DNA) has no oxygen molecule on the second carbon of this ring, while ribonucleic acid (RNA) does. Furthermore, DNA has the nitrogen base thymine, while RNA has uracil. In 1950, it was observed by Chargaff that the total amount of pyrimidine and purine bases were equal and that the amount of A was equal to T and G was equal to C; this is now known as Chargaff's Law [Chargaff et al, 1950; Southin, 1991a]. A nitrogen base bound to a ribose or deoxyribose pentose ring is called a nucleoside, while the further addition of a phosphate group to this complex is a nucleotide (ie: CTP). In 1953, using Chargaff's Laws and the X-ray diffraction studies of Wilkins and Franklin, Watson and Crick used the 'paper cut-out model' to demonstrate that DNA is a double-stranded, helical structure whereby the two strands are of complementary nucleotide sequence (*i.e.*; if one strand reads AGC, the other would be TCG) [Crick et al, 1954; Southin, 1991c]. These strands are held together by spontaneously forming hydrogen bonds, 2 between the A and T bases, and three between G and C (Diagram 2.2).

2.4b Transcription

Although the instructions for protein production are found within the sequence of DNA, it is the messenger RNA (mRNA) that conveys this information to the 'protein synthesis' machinery. During transcription the cell uses the DNA as a template for the synthesis of RNA. Althgouh there are a number of different types of RNA, it is the messenger RNA (mRNA) that codes for secreted protein. Synthesis of mRNA is



Double stranded DNA
anti-parallel

A≡T *2 hydrogen bonds*

G≡C *3 hydrogen bonds*

Diagram 2.2 DNA is double stranded and anti-parallel in sequence

initiated with RNA polymerase II (pol II), which binds to the gene's promoter site and induces local unwinding and separation of the two DNA strands. This promoter sequence is found on only one strand of the DNA, the template strand, and its activation initates the addition of monophosphate ribonucleotides (ei; AMP) in the 3'-5' direction. As such, the nascent RNA is synthesized 5'-3' and is in anti-parallel orientation, or complimentary, to the template DNA. This event proceeds until pol II recognizes a termination sequence, at this point transcription is arrested [Alberts et al, 1994a]. Adenlyl residues (A) are added post-transcriptionally to the carboxyl end of the transcript by poly-A polymerase action and are thought to increase mRNA stability by protecting against ribonuclease degradation [Hawkins, 1991a]. This unprocessed pre-mRNA transcript consists of both coding and non-coding regions, refered to as introns and exons, respectively. Once the poly A tail is added, the transcript undergoes extensive processing by nuclear enzymes which cut out the intronic regions and splice the exons together end to end, forming the mature mRNA transcript. After splicing is complete, the mRNA moves out of the nucleus and engages with a ribosome for translation [Hawkins, 1991a; Alberts et al, 1994a; 1994b].

2.4c Translation

As mRNA exits the nucleus, it binds to the ribosome and is ready to direct protein synthesis. The information encoded within the mRNA transcript is stored in the form of a triplet code. Each triplet or codon, consists of three ribonucleotide bases and codes for a single amino acid. Although 64 codons can arise from permutation of the four bases (4^3), only 20 amino acids are incorporated into protein. This is attributed to

some amino acids being coded for by more than one codon and also indicates the degenerate nature of the code. The complimentary triplets are known as anti-codons and are found on transfer (t) RNA. The tRNA is co-valently linked with one of at least 20 different aminoacyl-tRNA synthetases, which catalyze the linkage of the amino acid to a particular tRNA. The tRNA bearing the attached amino acid is then linked to the mRNA codon for that amino acid [Hawkins, 1991b; Alberts et al, 1994c].

Initiation of protein synthesis occurs when the AUG codon on the mRNA transcript binds to a tRNA molecule carrying the amino acid methionine (tRNAmet). The polypeptide chain is elongated by the successive addition of other amino acids, at a rate of about 2-3 per second. When the ribosome reaches a termination codon in the mRNA, the link between the polypeptide chain and the last tRNA is broken and the protein is released from the ribosome [Alberts et al, 1994c].

2.5 General principles of *in situ* hybridization

The technique of *in situ* hybridization is based on the concept that a synthesized DNA or RNA polynucleotide probe will hybridize with the DNA or RNA sequence of interest within the cell. There are a number of techniques, such as Northern blot analysis and polymerase chain reaction that utilize the principle of complementary sequence hybridization. The strength of *in situ* hybridization, however, is the fact that it is performed on sections of whole tissue, not homogenates, and therefore the signal can be localized to particular cell types. Initially, probes used in *in situ* hybridization experiments were generated to detect DNA [Pardue et al, 1969], however, this technique is now commonly employed for identifying particular mRNA transcripts.

This method is often more informative, since the presence of mRNA indicates that gene transcription has taken place. The presence of the mRNA in question is identified by its hybridization with an anti-sense probe, which is labeled for visualization. There have been different reports on the sensitivity of ISH, however, it is commonly accepted that this technique reliably detects 100 or more copies. There are a variety of different types of probes used for the intracellular detection of mRNA.

2.5a Types of Probes

Double- and single-stranded DNA, oligonucleotide and single-stranded RNA sequences have all been used to detect mRNA. Double-stranded DNA probes are produced by bacterial amplification of an inserted DNA sequence [Singer et al, 1986; Morimoto et al, 1987]. Single stranded DNA probes can be generated from the bacteriophage M13, by reverse transcription of RNA or by amplified primer extension of a PCR-generated fragment [Herrington et al, 1990a; 1990b]. The use of DNA probes is less desirable, however, since they are subject to re-annealing. Oligonucleotide probes are highly useful if the amino acid sequence, but not the cDNA itself, is readily available to the investigator [Herrington et al, 1990a; 1990b]. They are generated by automated synthesis, usually of 20 - 40 base pairs in length. Since they are very short, they do not confer the sensitivity of longer probes, however, Thein et al have demonstrated that oligonucleotides of 16 or more base pairs are unique in the human genome [Thein et al, 1986]. The fact that they are so short also holds its advantages, as they penetrate the cell easily.

Single-stranded riboprobes were used for the majority of this thesis work, and are generated by adding nucleotides, RNA polyermases and a vector-inserted cDNA template together for *in vitro* transcription of RNA fragments. The advantages of using these probes are multi-fold. RNA:RNA hybrids are highly stable, which allows for the use of stringent conditions such as high temperature and salt concentrations to reduce non-specific probe binding. Furthermore, unlike their single-stranded counterparts, these hybrids are Rnase resistant, which makes an ideal method for eliminating unhybridized probe, diminishing background signal. Furthermore, these riboprobes are quite sensitive, as they can be synthesized to a length ~ 500 bp, allowing the label to be incorporated along the entire span of the probe [Wilcox et al, 1993; Hamid et al, 1997b].

2.5b Probe Construction

To generate RNA probes complementary to mRNA, cDNA is inserted into an expression vector with promoter regions that recognize RNA polymerases. This vector:cDNA unit is subcloned in bacteria and then linearized using the appropriate restriction enzymes. Bluescript and pGEM are the most common vectors used for generating RNA probes, since they have multiple promoter regions which flank the DNA insert and allow for transcription to take place in either a 5'-3' or a 3'-5' direction, thereby producing fragments that are either anti-sense (complementary to mRNA) or sense (identical to mRNA; Diagram 2.3).

2.5c Probe Labels

There are two distinct types of label used for these probes: radiolabeled and non-radiolabeled. Radio-isotopes frequently used for *in situ* hybridization include ^{32}P, ^{3}H and ^{35}S. With these labels, hybridization signal can be detected by autoradiographic liquid emulsion or X-ray film. ^{32}P is a highly energetic β emmitter, with a half life of 14 days and requires only a 2-3 day exposure before analysis. These high energy particles travel quite far through autoradiographic emulsion producing a wide scattering of silver grains, which reduces resolution and mask morphology. Nonetheless, this label is highly useful for rapid analysis when cellular detail is not essential. ^{3}H is a low-energy radio-isotope with a half life of 12 years. It confers much better resolution than ^{32}P, excellent for morphological analysis, however, the average exposure time is in the range of 2-3 months. The label used for the majority of this thesis work was ^{35}S, which has a half life of 87 days and gives off particles of intermediate energy on decay. This label requires 8-15 days of exposure, has medium resolution and allows for adequate visualization of cellular and subcellular detail. There are distinct advantages to using radiolabeled riboprobes, including the fact that radioisotopes are readily incorporated into the *in vitro* synthesized RNA and autoradiography represents the most sensitive of detection systems. Nevertheless, through efforts to develop a non-radioactive method of *in situ* hybridization, the use of non-isotopic labels has become available. The first of these was biotin -[d]UTP, a biotinylated nucleotide analogue of dTTP [Langer et al, 1981; Giaid et al, 1989]. It has been demonstrated that some tissues produce biotin,

like the liver, heart and skeletal muscle [Niedobitek et al, 1989; Kirkeby et al, 1993], and as such this label is not always the best choice. Fluorescent probes, generated by

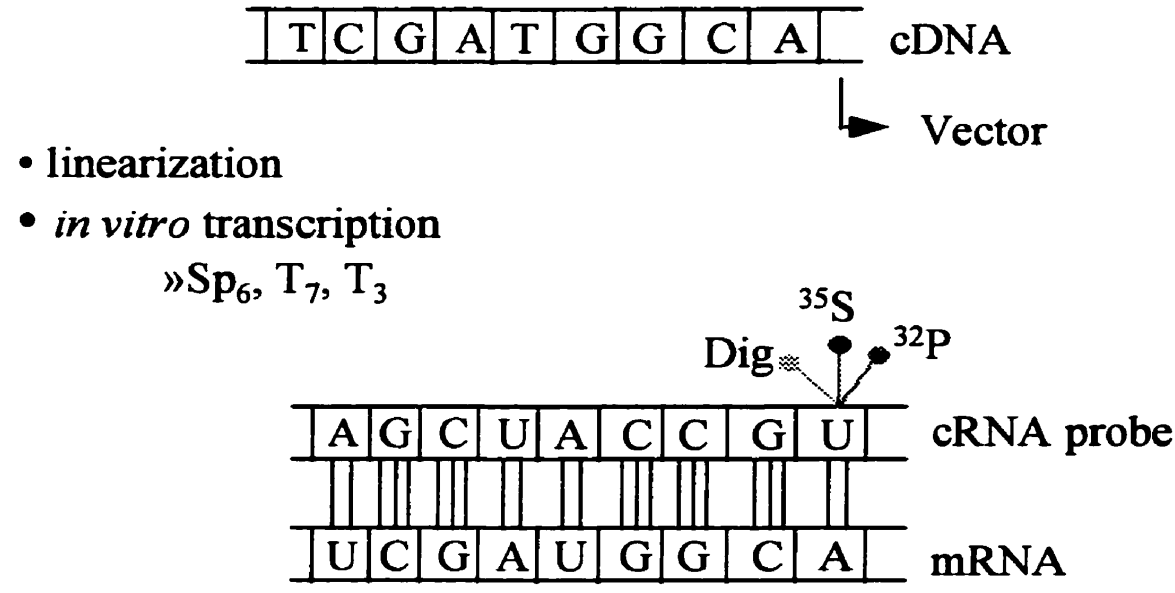


Diagram 2.3 *In vitro* transcription and labelling of riboprobes for the detection of mRNA.

tagging the probe with a fluorochrome such as fluorescein isothiocyanate (FITC), Texas Red [Larramendy et al, 1998] and rhodamine [Bauman et al, 1985], are also used. These labels give high sensitivity, however, they do not provide morphological information. Furthermore, since the fluorescent compounds fade within weeks it is necssary to record observations photographically. The current method of choice for non-radioactive probe labeling is digoxigenin [Heino et al, 1989; Ying et al, 1993]. Digoxigenin is a steroid isolated from the digitalis plant, *Digitalis purpurea* or *Digitalis lanata.* The blossoms and leaves of these plants are the only natural source of digoxigenin and as such detection methods are not susceptible to its endogenous, as with biotin. Dig-labeled hybrids are detected using an alkaline phosphatase-conjugated antibody raised against digoxigenin (Dig-AP) and color development. ***In situ*** hybridization with the DIG-AP system *vs.* the ^{35}S labeled riboprobe system have been reported to have similar sensitivity [Miller et al, 1993; Diagram 2.3].

2.6 Protocols used for *in situ* hybridization

2.6a Probe construction

In this thesis, anti-sense and sense RNA probes were generated for the ε germline gene transcript (Iε), ε heavy chain (Cε), IL-4 and IL-13 mRNA. The Iε probe (Diagram 2.4) corresponded to the start site of the promoter sequence, referenced as nucleotide 1, to the end of the Iε exon at position 650. The cDNA was cut using the Bam HI restriction enzyme and subcloned into the Bluescript Ksp vector. After cloning, the plasmid was linearized with Xba I or Hind III restriction enzymes [Gauchat et al, 1990]. This linearized fragment was used for *in vitro* transcription, in the presence of

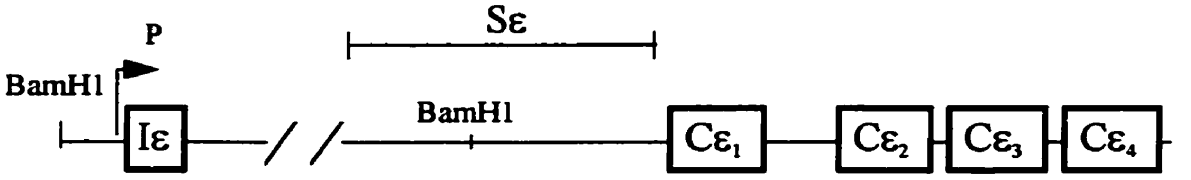
[^{32}P]-UTP or [^{35}S]-UTP with T7 RNA polymerase for the antisense probe and T2 RNA polymerase for the sense probe [Melton et al, 1984].

The Cε probe was generated from an ε chain cDNA clone [Kenten et al, 1982]. The sequence corresponded to a 1150-base Hind III fragment from pSC213, encompassing 24 bases of Cε1 and the rest of the Cε sequence (Diagram 2.3). This fragment was cloned into a pGEM vector, cut with Sma I restriction enzymes and transcribed with in the presence of [^{35}S]-UTP with Sp6 RNA polymerase for the antisense probe (526 nucleotides) or cut at the Bam HI site and transcribed with T7 RNA polymerase for the sense probe.

The IL-4 probe was generated from a human 500bp fragment (generously provided by Dr. J.-Y. Bonnefoy, Glaxo Institute for Molecular Biology, Geneva, Switzerland). It was inserted into pGEM, linearized by Sph1 and EcoR1 restriction enzymes and transcribed in the presence of ^{35}S-UTP with T7 or Sp6 polymerase for the antisense and sense probes, respectively.The sequence for the IL-13 probe (a kind gift from Dr. A. Minty, Sanofi Recherche, Labege, Cedex, France) corresponded to a 1282bp fragment. It was inserted into Bluescript and linearized with Not 1 and BamH1 restriction exzymes. Sense and antisense probes were generated by *in vitro* transcription using T3 and T7 RNA polymerases and the probe was labeled by ^{35}S-UTP incorporation.

A consensus oligonucleotide probe for Cγ, with complete homology to all four IgG subclasses, was generated by automated synthesis. The sequence corresponded to the Cγ_2 region (IgG_1, nucleotide 1206-1235 of accession no. J00228; IgG_2, nucleotide

Genomic DNA



RNA precursor

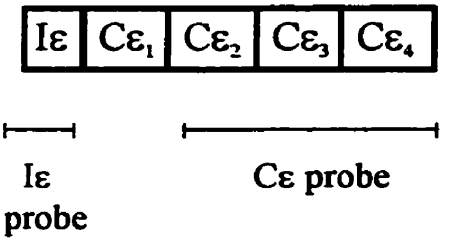


Diagram 2.4. Identity of the Iε and Cε probe. Top line: show sites of digestion with BamH1, the start site of transcription (P), the switch region (Sε) and the Iε exons encoding the four ε chain constant regions (Cε_1-Cε_4). Middle line: the unprocessed ε germline gene transcript. Bottom line: the mature RNA ε germline transcript after RNA splicing, and the sequences corresponding to the Iε and Cε probes.

1204-1233 pf J00230; IgG_3, nucleotide 1785-1814 of X03604; IgG_4, nucleotide 1205-1234 of K01316). It was either 5' end labeled with ^{32}P UTP or with the non-isotopic label digoxigenen 11-UTP.

2.6b Radiolabeled in situ hybridization [Hamid et al, 1987; Hamid et al, 1997b]

2.6b.i *In vitro* transcription of radiolabeled probes

To perform *in vitro* transcription of radio-labeled probes, 1.0 ug/ml of linearized plasmid template, (either Cε, Iε, IL-4 or IL-13), was added to 10 µl mixture of 5X transcription buffer (200mM Tris-HCl; pH 7.5, 30 mM $MgCl_2$, 10mM spermidine, 5 mM NaCl), 100 mM diethylthreitol (reduces non-specific binding), 25 U/µl of ribonuclease inhibitor (arrests degradation of the RNA product), nucleotide mixture (2.5 µM of ATP, GTP, CTP), 25 mCu of ^{35}S-UTP and 10 units of RNA polymerase (T7, Sp6 or T3) and heated to 37°C in a waterbath for 60 minutes. The polymerase catalyzes transcription from its corresponding promoter on the vector and the nucleotides bind to the cDNA in complimentary fashion. Rnase-free Dnase is added to separate the RNA probe from the cDNA template, leaving single-stranded RNA fragments. To extract the probe from unincorporated nucleotides, 10 µg/ml of tRNA, 4M NaCl and phenol-chloroform (1:1, v/v) were added. After microfugation at 12,000g, the aqueous phase was removed and subjected to a second extraction with an equal volume of chloroform. The aqueous phase was treated with 100 µl of 7M ammonium acetate (2.5 M final concentration) and 750 µl of cold (stored at -20°C) absolute ethanol and left to precipitate overnight at -20°C or for two hours at -80°C. This mixture was microfuged at 4°C, the aqueous phase removed, except the last 50µl,

which was placed in a speed vacuum centrofuge for dissipation. The remaining pellet, the purified RNA probe, was dissolved in 20 µl of DEPC-treated water. Incorporation of the radiolabel was assessed using a β-emmissions counter by placing a 1 µl sample of the probe into 5 ml of scintilating cocktail, in this manner the total counts per million (cpm) were measured. ^{35}S-labeled probes used for these experiments were always $\geq 1.0 \times 10^6$ cpm.

2.6b.ii Pre-hybridization

Pre-hybridization of the tissue sections is important for permeabilization of the cell membranes, to allow for passage of the riboprobe to the interior of the cell for hybridization with mRNA. Slides were immersed in 0.1M glycine/PBS and then in a 0.3% Triton-X-100/PBS solution, which degrades the cell surface proteins, for 5 minutes. Slides were then placed in a 1µg/ml proteinase K solution, dissolved in 1M Tris-HCl pH 8.0 and 0.5M EDTA and PBS, for 20 minutes at 37°C to degrade intra-membrane proteins. Further permeabilization and fixation was performed by placing the slides in a freshly prepared solution of 4% PF/PBS for 5 minutes. Pre-hybridization also includes steps for reducing non-specific binding. These were carried out by immersing the slides in 2 high salt solutions, of *1)* 0.25% acetic anhydride and 0.1M triethanolamide and *2)* 0.1M N-ethylmalamide and 0.1M iodoacetamide both dissolved in distilled water, and used at a temperature of 37°C for 10 minutes and 20 minutes, respectively. Immersion in a mixture of 50% ionizing formamide in 4X standard saline-citrate (SSC) for 15 minutes at 37°C was used to regulate tissue equilibrium and stabilization. Finally, slides were dehydrated by placing them in increasing

concentrations of ethanol (70%, 90% and 100%) for 5 mintues each and left to dry for a period of at least 2 hours.

2.6b.iii Hybridization

For optimal cRNA:mRNA hybridization, a probe mixture was prepared from hybridization buffer (90% total volume) consisting of 50% deionizing formamide, 5X Denhardt's solution, 10% dextran sulphate, 0.5% sodium pyrophosphate, 0.5% SDS, and 100mM dithiothreitol (DTT). To the buffer, 0.75 X 10^6 cpm/section of radiolabeled riboprobe (Cε, Iε, IL-4 or IL-13), was added with 10 mM of DTT. Each slide was incubated with 15µl of this probe mixture and covered with dimethyldichlorosilane-coated coverslips. Slides are hybridized in a humid chamber, created by pouring SCC solution (a 2cm depth) in the bottom of the slide box, and incubated overnight at 42°C. It is important to use the humid chamber, otherwise this relatively high temperature will evaporate the probe mixture.

2.6b.iv Post-hybridization

All post-hybridization washes were performed for 20 minutes at 42°C by placing the solution dish and slide rack in a water bath of this temperature. Slides were first dipped individually in 4X SSC for gentle removal of the coverslip and then placed in three successive washes of 4X SSC. These are considered stringent conditions, since both the temperature and salt concentrations were relatively high; both effective methods for reducing non-specific charge-based binding. A further step taken to minimize background was to eliminate the excess unhybridized probe. This was done by treating slides with 20µg/ml of Rnase A dissolved with 4M NaCl, 1M Tris, and

0.5M EDTA. Rnase A will degrade the single, but not double-stranded, RNA. Following this, sections are put through decreasing concentrations of SSC washes of 2X, 1X, 0.5X and 0.1X SSC for 20 minutes each and then dehydrated in 70%, 90%, and 100% ethanol solutions with 0.3% acetic anhydride, for 10 minutes each.

2.6b.v Autoradiography

To visualize the cells containing mRNA-cRNA radio-labeled hybrids, slides were dipped in liquid emulsion in a dark room under red light and left overnight to dry in complete darkness. Exposure took place in a light-proof sealed black box at 4°C for a period of 10-15 days. Slides were developed under red light using Kodak D-19 developing solution at 16-18°C for 3.5 minutes with gentle agitation. This reaction was arrested by placing the slides in water. Fixation of the development was performed by immersing the slides in Rapid Fixer solution for 5 minutes at 16-18°C and then rinsed for 20 minutes in water. Using a straight razor blade, excess emulsion was removed and special care was taken to clean the underside of slide beneath the tissue. Slides were counter stained in hematoxylin, a blue basic dye that combines with acid substances and therefore stains the nuclei, creating a constrast for histological analysis with light field microscopy. Slides are dehydrated in increasing concentrations of ethanol (70-100%) and finally immersed in xylene. Subsequently, slides were coverslipped directly by placing a dab of entellyn, which adheres the coverslip to the slide. Under light microscopy, positive signal is observed as a cluster of small black silver grains overlying the cell.

2.6b.vi Controls

Negative control experiments were performed using either sense probes or RNase pre-treatment. The sequence of a sense probe is identical to that of the mRNA and therefore replacing the anti-sense with a sense probe in the hybridization mixture should result in no positive signal. A second control performed was to pre-treat the tissue with an 100 μg/ml of RNAse A at 37°C, which digests all single stranded RNA, and then proceed with the hybridization protocol.

2.6c Non-radioactive in situ hybridization [Ying et al, 1993]

2.6c.i *In vitro* transcription of non-radiolabeled probes

Non-radiolabeled cRNA probes were also generated using *in vitro* transcription, in a similar fashion as the radio-labeled procedure. To do this, 1.0 μg/ml of linearized plasmid template, in this case the Cε cDNA, was added to a 20 μl mixture of 5X transcription buffer (200 mM Tris-HCl; pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 5 mM NaCl), 100 mM diethylthreitol, 25 U/μl of ribonuclease inhibitor, 20 units of RNA polymerase (T7, Sp6 or T3) and 8 μl of nucleotide mixture, consisting of 10 μM each ATP, GTP, CTP, and 6.5 μM UTP and 3.5 μM digoxigenin-11-conjugated UTP. This mixture was heated to 37°C in a waterbath for 2 hours. Following which, 1 μl of RNase-free Dnase was added for 15 minute incubation a waterbath at 37°C. The probe was precipitated in 4 M NaCL and cold ethanol overnight at -20°C. The next day, this mixture was centrifuged at 12, 000 g at 4°C for 10 minutes. The supernatant was discarded and the remains were washed in 100μl of 70% cold ethanol, dried under vacuum and dissolved in 20 μl of DEPC H20. The probe was placed at 65°C for 5

minutes to help it dissolve and then stored at -20°C. The concentration of these probes was determined by running it on a 2% gel in 0.5 x TBS.

2.6c.ii 3' end labeling of oligonucleotide DNA probes [Schmitz et al, 1991]

To detect the expression of Cγ mRNA, an oligonucleotide probe was 3' end-labeled with digoxigenin. To do this, 100 pmol of Cγ oligonucleotide were added to 20µl mixture of 4 µl reaction buffer (1 M potassium cacodylate, 0.125 M Tris-HCL, 1.25 mg/ml Bovine serum albumin, pH 6.6 at 25°C), 25 mM $CoCl_2$, 1 mM Dig-ddUTP and 50U Terminal Transferase in 200 mM potassium cacodylate, 1 mM EDTA, 200 mM Kcl, 4 mM 2-mercapto-ethanol, 50% glycerol, pH 6.5 (4°C). This mixture was incubated at 37°C for 15 minutes and placed on ice. The reaction was arrested by adding a 2 µl solution of glycogen/distilled water (20mg/ml) and 0.2 M EDTA; pH 8.0. Oligonucleotide was precipitated with 2 mM LiCl and prechilled (-20°C) ethanol and left for 30 minutes at -80°C or 2 hours at -20°C. This mixture was centrifuge at 12, 000 g, the pellet was washed with cold 70% ethanol, dried under vacuum and dissolved in DEPC treated water. This probe was stored at -20°C until use.

2.6c.iii Pre-hybridization

The protocol for non-radiolabeled *in situ* hybridization is based on the radiolabeled method and as such is relatively similar. Slides were brought to room temperature, placed in a 4% PF in PBS solution for 5 minutes and then in 0.3% Triton X-100 in PBS for 5 minutes. Sections were then immersed in a proteinase K solution (1 µg/ml in 20 mM Tris-HCl and 1 mM EDTA, pH 7.2) for 15 minutes at 37°C. Finally, a

second immersion in 4% PF in PBS for 15 minutes was performed and slides were left to air dry for 1 hour.

2.6c.iv Hybridization

A hybridization mixture was prepared containing hybridization buffer (90% of total volume), 50% formamide, 5X Denhardt's solution, 5 x SSC and 0.5 µg/ul denatured salmon sperm DNA. To this mixture, 250 ng/section of digoxigenin-labeled probe was added. Each section was incubated with 20 µl of the hybridization mixture and they were incubated overnight in a humid chamber at 40°C.

2.6c.v Post-hybridization and development

The following day, slides were placed in 2 washes of 4X SCC at 42C for 10 minutes and then incubated with 20 µg/ml RNAse A in 2 x SSC for 30 minutes at 37°C. Washes were completed with 2 x SSC at 42°C for 10 minutes followed by 0.1 x SSC wash for 10 minutes at room temperature. After a brief wash in TBS, sections were incubated with 3% bovine serum albumin/TBS for 10 minutes to reduce non-specific background then incubated with 40 µl/section of a 1:1000 dilution of Dig-AP conjugated (sheep polyclonal anti-digoxigenin anti-serum conjugated with alkaline phosphatase) in TBS containing 0.1% Triton X-100. This incubation period was performed in a humid chamber for 4 hours at room temperature. Following this, slides were washed 3 times (15 min each) in TBS and then incubated in an equalization buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50mM MgCl2, pH 9.5) for 5 minutes. Color development was achieved by adding a freshly prepared substrate solution consisting of 0.350 mg X-phosphate-5-bromo-4-chloro-3-indoly phosphate (BCIP) and 0.740 mg

nitroblue tetrazolium (NBT) salt per ml of equalization buffer to the slides for 40-50 minutes at room temperature. Light microscopy was employed to determine the optimum reaction time. The reaction was terminated by the washing the slides in TBS and then tap water. Counterstaining was performed with hematoxylin for 5 s and the slides were then mounted with crystal mount. Under light microscopy, positive signal is observed as a purple staining of the cells.

2.7d Simultaneous in situ hybridization [Kotsimbos et al, 1996]

Simultaneous *in situ* hybridization was performed with probes coding for Cε and Iε by labeling the Cε probe with digoxygenin and the Iε probe with ^{35}S. Sections underwent the pre- and post-hybridization protocol for digoxygenin *in situ* hybridization, however, the hybridization mixture contained both riboprobes and was incubated overnight at 42°C. The Cε probe was visualized according to the mehthods described for single digoxigenin *in situ* hybridization. Slides were subsequently dipped in liquid emulsion, exposed for 7 days and developed according to the protocol for radio-labeled *in situ* hybridization to visualize the hybrized Iε probe. Double positive cells were associated with both a purple staining for the digoxygenin-labeled Cε and clusters of silver grains, identifying hybridization of the Iε probe.

2.7 General principles of immunocytochemistry

Immunocytochemistry employs the principles of immunology to localize tissue- or cell-associated antigens by means of a labeled antibody. The concept of using antibodies to detect antigen within tissue is credited to A.H. Coons and colleagues [Coons et al, 1941], which sparked the interest of the scientific community and resulted in the

development of what is known today as *immunocytochemistry*. This technique has become highly useful in the field of diagnostic histopathology, aiding in the diagnosis of tumors, identification of foreign organisms as well as the development of autoimmune diseases. Furthermore, it is also now widely used in experimental work to monitor the presence of particular cell types, using antibodies to detect phenotypic markers, cell products like hormones and cytokines as well as their receptor expression. While different methods can be employed to improve the quality of results, the most important aspect of this technique is the development of antibodies which bind to the antigen of interest.

2.7a Types of Antibodies

Antibodies for immunocytochemistry can be one of two types, polyclonal or monoclonal, both of which are used in this thesis work. The processes involved in generating these antibodies are briefly described below. In many cases, however, it has become standard practice to purchase antibodies from a biotechnology company rather than raising them in-house.

Polyclonal antibodies are generated by immunizing an animal (rat, rabbit, hamster, goat, pig, horse etc) with a small amount of the antigen one wishes to detect, initiating an immune response. The animal will then produce antibodies with different specificity and binding affinity for the particular antigen [De Mey et al, 1986], which can be obtained by routine bleeding. The sera of an immunized animal contains a mixture of antibodies, recognizing different epitopes of the immunizing antigen. As such, these antibodies are considered highly sensitive. However, disadvantages in using them

include the fact that the supply is limited to the serum of one animal and that antibody-specificity has been shown to change substantially from one animal to another, even when immunized with identical antigen [Vandesande et al, 1979]. These difficulties are largely responsible for the development of monoclonal antibodies.

Unlike polyclonal antibodies, monoclonal antibodies (mAbs) are specific for only one epitope of the antigen [Milstein et al, 1979]. To produce them, mice are immunized with the antigen of interest, the B cells are removed and cultured with immortalized myeloma cells, taken from the same strain of mouse. The progeny of this population are referred to as hybridomas. They proliferate indefinitely and produce antibodies specific for a variety of epitopes, as polyclonal anti-sera does. To generate monoclonal antibodies, however, a single B cell is isolated from these hybridomas and clonally expanded. The entire population of B cells which arise from this single cell produce antibodies with identical epitope specificity. Since these cells proliferate *ad infinitim*, the problem of cross-batch or cross-animal irregularities, as seen with polyclonal antibodies, is eliminated. Furthermore, an added benefit is that the cells can be frozen and re-stimulated at a later date. Using monoclonal antibodies is less sensitive than using polyclonals, since they recognize the same epitope. This can be overcome by using a mixture of monoclonal antibodies which recognize different epitopes of the same antigen or with amplification of detection methods [Hamid et al, 1997c].

2.7b Different methods for immunocytochemistry

Immunocytochemistry can be performed by a number of different methods. These include *a)* the *direct method*, which entails applying a labeled primary antibody that binds a specific antigen; *b)* the *indirect method* uses an unlabeled primary antibody and detection by a labeled second layer antibody, raised against the primary; or *c)* the *enzyme-linked method*, a modification of indirect immunostaining, that uses an unconjugated bridging antibody between the primary and the enzyme-complexed tertiary antibody [Hamid et al, 1997c].

2.8 Protocols used for immunocytochemistry

2.8a Alkaline phosphatase anti-alkaline phosphatase technique [Frew et al, 1988]

The majority of immunostaining performed in this thesis was done using the alkaline phosphatase anti-alkaline phosphatase (APAAP) enzyme-linked method, which is a modified version of that described by Cordell [Cordell et al, 1984]. Sections were hydrated in Tris buffered saline (TBS) solution and incubated with a mixture of commercially available non-specific blocking antibodies for 10 minutes. Mouse monoclonal antibodies directed against cluster determinant (CD)20, to identify B cells, were diluted to a final concentration of 1/30 with an antibody diluting buffer. Sections were incubated with 45-60 µl of primary antibody solution in a humid chamber overnight at 4°C. The following day, slides were washed for 6 minutes (2 washes for 3 minutes each) in TBS and incubated with a secondary layer consisting of rabbit anti-mouse polyclonal IgG (1/60 dilution) at room temperature for 30 minutes. After this, slides were washed in TBS for 6 minutes and the tertiary layer of alkaline-phosphatase

(AP)-conjugated rat anti-rabbit polyclonal IgG (1/60 dilution) was applied for 30 minutes. To visualize the cells binding this antibody-AP complex, sections were incubated with a mixture of AP substrate and Fast Red TR chromogen (0.5 mg/ml). When the enzyme/substrate reaction takes place the red chromogen precipates onto the cells linked to AP. In the case of CD20, the second and third layers were repeated to enhance detection of this antigen.

2.8b Double immunocytochemistry

Double immunocytochemistry was performed to determine the expression of IgE by B cells (CD20), plasma cells (CD38) and mast cells (tryptase). To do this, a combination of the APAAP and the horse raddish peroxidase (HRP) methods were employed. First, endogenous peroxidase activity was blocked by incubating the sections with a 1% H_2O_2 solution for 10 minutes. The slides were washed for 6 minutes in TBS and incubated with blocking solution for 10 mintures. The mouse anti-human antibodies against CD20, CD38 or tryptase were applied at a dilution of 1/30 and the rabbit anti-human IgE was 1/200. The slides were then placed in a humid chamber overnight at 4°C. The next day, slides were washed in TBS for 3 minutes and the second layer was applied for 30 mintues at room termperature. This consisted of rabbit anti-mouse (1/60) and biotinylated swine anti-rabbit (1/200) antibodies. After a 3 minute wash in TBS, slides were incubated with a teriary layer of rat anti-rabbit-AP conjugated antibodies (1/60) and a complex of rabbit streptavidin-biotinylated HRP (1/500) for 30 minutes. After washing in TBS, positive signal was visualized by incubating the sections with a mixture of AP substrate and Fast Red TR chromogen (0.5mg/ml) as

well as diaminobenzidine (DAB; 0.3 mg/ml) and 30% H_2O_2. The enzyme/substrate reactions induced the precipation of Fast Red particles onto those cells complexed to AP, and DAB onto those cells complexed to HRP. As such, cells expressing the phenotypic markers CD20, CD38 and tryptase stained red while the IgE+ cells appeared brown. Those cells that were double positive stained both colors and was observed as a reddish brown.

2.9 Simultaneous immunocytochemistry and *in situ* hybridization.

To phenotype the cells expressing various mRNA products, simultaneous immuncytochemistry and *in situ* hybridization was performed. In this thesis work, two methods of immunostaining have been utilized for this simultaneous technique, HRP and APAAP. In an attempt to determine the proportion of B cells expressing ε chain (Cε) and germline gene transcripts (Iε), double immunocytochemistry and *in situ* hybridization was performed. To do this, CD20 was stained using either the HRP or APAAP method, as described above, and stained brown or red, respectively. The slides were then processed for ISH with radio-labeled riboprobes complimentary to Cε and Iε RNA following the protocol detailed in section 2.6b (p. 100). In a similar manner, with APAAP immunocytochemistry and radio-labeled ISH, the proportion of CD3 (T cells) and tryptase (mast cells) immunoreactive cells expressing IL-4 and IL-13 mRNA was also determined. Under light field illumination, double positive cells appeared as brown or red, according to the staining method, with collections of black silver grains overlying the cells.

2.10 Quantification

In situ hybridization and immunocytochemistry are sensitive and specific techniques. In either case, however, it is not possible to quantify the actual number of mRNA copies or protein molecules detected. Quantification using these techniques is done primarily through reporting the *number of cells* that are expressing the mRNA transcript or protein in question, commonly referred to as point counting. To do this, light field illumination with a grid attachment used. At a power of 200X, the number fo number of positive cells per field were counted. The mean number of cells was calculated for 4-6 fields and inter-observer variability was < 10%.

2.11 Statistics

Analysis was performed using a statistical software package from Jandel Scientific (Systat Statistical Software version 7.1) with p values less than 0.05 considered significant. Within group comparisons (baseline *vs.* allergen challenge/exposure) were analyzed using the Wilcoxon matched-pairs signed ranks test. For between group comparisons (placebo *vs.* steroid or allergci *vs.* non-allergic), the delta value *i.e,* the difference in the number of positive cells before and after allergen exposure, was calculated and an unpaired Student's t test was applied to these values. Correlations were performed using Spearman's rank method.

Chapter 3

3.0 RESULTS

3.1 *In vivo* allergen challenge

3.1a Clinical response after in vivo allergen challenge.

Patients were well matched for age, gender and circulating grass pollen-specific IgE concentrations. The demographic profile of this patient group is seen in Table 3.1. Nasal symptoms following acute allergen challenge were recorded as the number of sneezes, weight of secretions and degree of nasal blockage. Allergen provocation resulted in both an early (within the first 60 minutes) and late (1-24 hours) onset of these symptoms. The mean number of sneezes observed within the first 60 minutes following allergen challenge was 16.5 (ranging from 0-30) in the placebo group, in contrast to a mean of 4.5 (0-12) in FP-treated patients ($p<0.01$). All but one placebo-treated patient experienced sneezes between 1-24 hours, a mean of 18.8 (0-40), while those pre-treated with FP exhibited none or very few during this time (2.4, 0-10; Figure 1A). The degree of nasal blockage was assessed by visual analogue scoring with values set from 0-10. This score was significantly higher in placebo-treated (5.9) than FP-treated (3.1; $p<0.01$) patients immediately following the challenge. In a similar manner, during the period of 1- 24 hours nasal blockage scores were also higher in placebo (5.5) compared to FP-treated patients (1.6; $p<0.05$; Figure 1B). The extent of rhinorrhea was assessed with pre-weighed tissue paper, which the patients used for clearance of nasal

Table 3.1 *Clinical characteristics of the patients that underwent in vivo allergen challenge*

	Treatment[a]	
	PL	FP
No. of patients	11	10
Age (yrs ± SD[b])	31.3 ± 2.7	30.0 ± 2.4
Gender (M:F)	6:5	4:6
Serum grass pollen-specific IgE (scale 0-5; mean ± SD)	2.7 ± 0.4	3.0 ± 0.3

[a]Patients received a six week pre-treatment with either placebo (PL) or fluticasone propionate (FP) nasal spray.
[b]Data is represented as the mean ± standard deviation (SD).

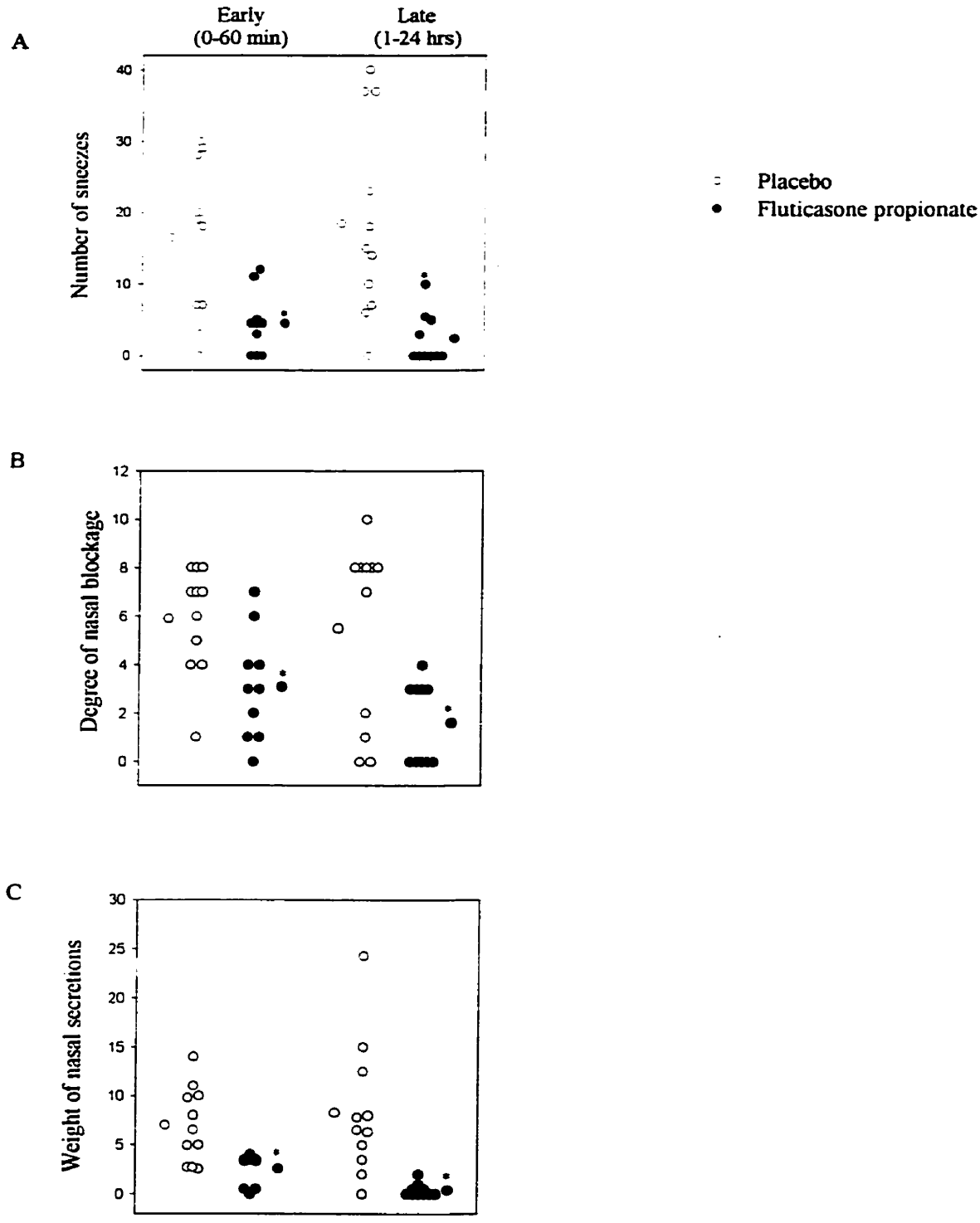


Figure 1. Clincal response measurements for analysis of *in vivo* allergen challenge. Data are presented as individual values after FP (n=10) or placebo (n=11) treatment. All symptoms (early and late) were inhibited by topical corticosteroid ($p<0.01$, except late blockage $p<0.03$).

discharge. The weight of nasal secretions collected during the early phase of the response was significantly more from patients that received placebo (7.0g), compared with those that were pre-treated with FP (2.6g, $p<0.01$). The mean weight collected during the time period of 1-24 hours following challenge from placebo-treated patients was similar (8.3g), however, FP treatment almost completely reduced the late onset of rhinorrhea in all patients, with nasal secrections weighing 0.4g (Figure 1C).

3.1b B cells within the nasal mucosa after in vivo allergen challenge.

B cells, identified using immunocytochemistry with monoclonal antibodies against CD20, were observed within the nasal epithelium and submucosal tissue. There was no significant change in the number of these cells within tissue obtained from placebo-treated patients before (median [range], 7[3-17]) and following (2[1-30]) *in vivo* allergen challenge. Similarly, patients that were treated with FP also exhibited no difference in the number of B cells (7[1-21] *vs.* 2[1-12]). CD20 is a cell surface protein which is implicated in B cell activation [Clark et al, 1986]. Consistent with this, the staining for CD20 immunoreactivity was membrane-associated. It is also interesting to note that the CD20+ cells were often found in clusters of two or three, indicating that inter-B cell signalling may occur (Plate 3.1a).

3.1c Expression of Cε and Iε RNA after in vivo allergen challenge.

Positive *in situ* hybridization signals for RNA product were observed as discrete collections of silver grains overlying the cells when anti-sense probes for $C\varepsilon$ and $I\varepsilon$ RNA were used.

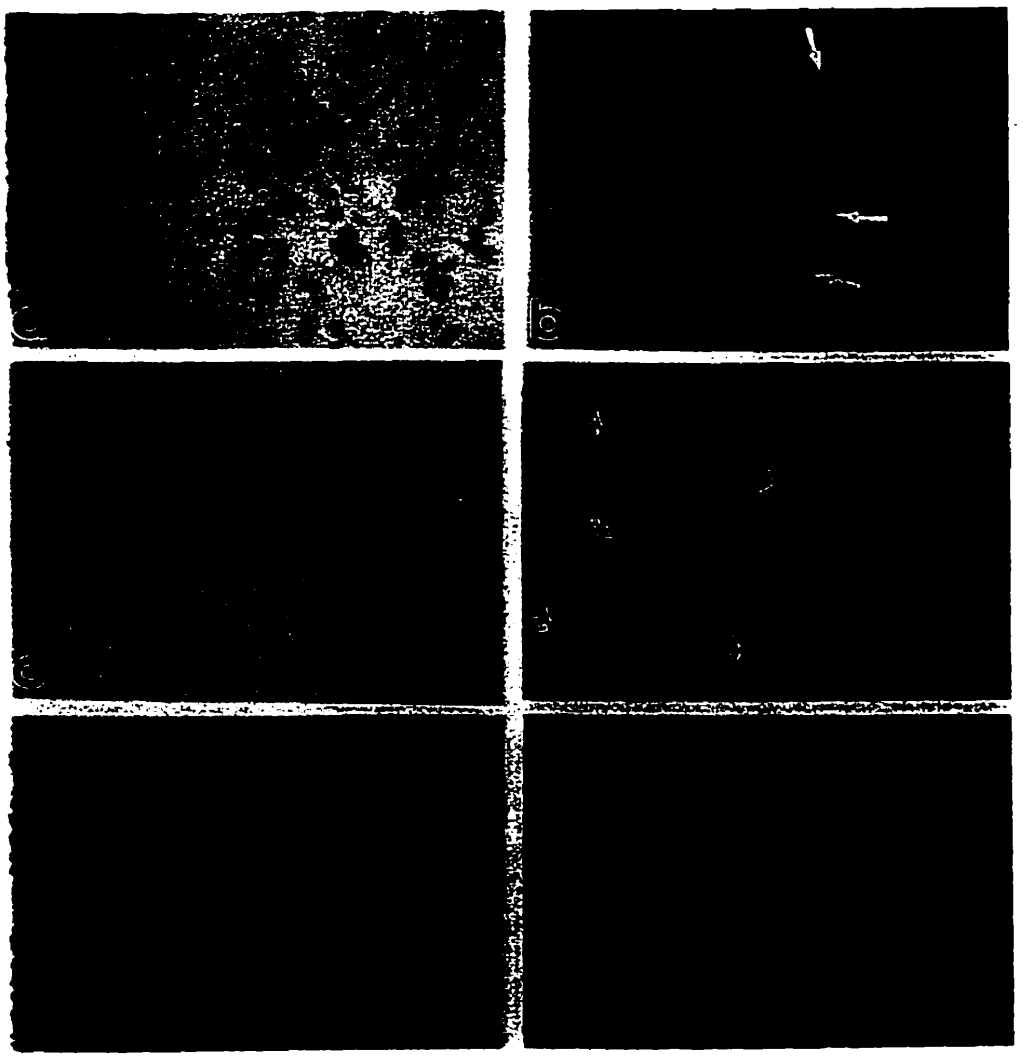


Plate 3.1 Immunocytochemistry and *in situ* hybridization demonstrating CD20+ B cells (a), IL-4 mRNA+ cells (b) and Cε RNA+ cells (c) co-expressing CD20 (d) within sections of nasal tissue obtained from patients after *in vivo* allergen challenge. Iε expression after allergen challenge is compared in patients pre-treated with placebo (e) or fluticasone propionate (f).

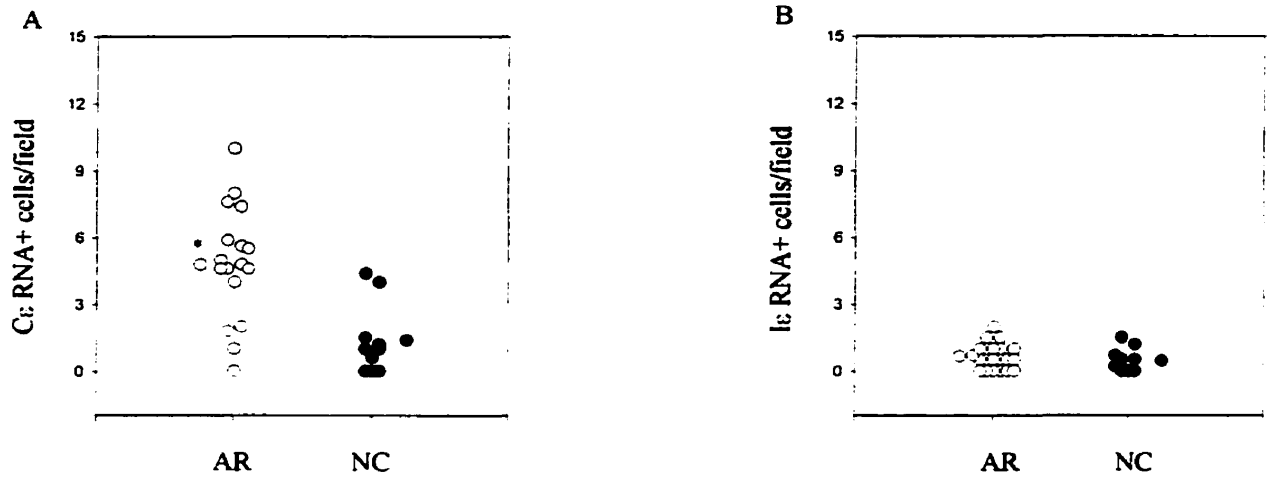


Figure 2: Comparison of ε gene transcripts within the nasal mucosa of patients with seasonal allergic rhinitis (AR) and non-allergic normal control (NC) subjects. There were more Cε RNA+ cells within allergic nasal tissue (n=17) obtained out of season, compared to control tissue (A; [n=10], $p<0.01$). The number of cells expressing Iε RNA were similar within allergic (n=21) *vs.* non-allergic (n=10) nasal tissue (B; $p>0.05$).

As expected, no specific positive signal was identified when sense probes were employed or when preparations were pre-treated with RNAse. To gain perspective on the baseline difference between in the expression of Cε and Iε RNA+ cells by seasonal allergic rhinitics, nasal tissue was obtained from these patients out of season and compared to that of normal control subjects. In both groups, there were virtually no Iε RNA+ cells (0.7 ± 0.6, 0.3 ± 0.4; Figure 2B). Although there were cells expressing Cε RNA within the nasal mucosa of both patients groups, the mean value was several times higher in seasonal allergic rhinitis patients (4.8 ± 2.6) than in normal controls (1.4 ± 1.6; Figure 2B).

When nasal tissue from patients pre-treated with matched placebo was examined 24 hours following allergen challenge, a marked increase in the number of Cε RNA+ cells (11.2 ± 7.2, Plate 3.1c) was observed compared to pre-challenge tissue (3.3 ± 1.6, $p<0.01$; Figure 3A) and cells expressing Iε RNA, those undergoing ε germline transcription, became clearly detectable (0.5 ± 0.4 *vs.* 5.1 ± 4.5, $p<0.01$; Figure 3B, Plate 3.1e). Furthermore, there was a significant positive correlation between the number of Iε RNA+ cells and the increase in Cε RNA+ cells ($r = 0.76$, $p<0.01$); *i.e.* the appearance of Iε RNA was similar to the increase in Cε RNA in individual patients. Simultaneous *in situ* hybridization with both Cε and Iε probes revealed that all Iε RNA+ cells were also Cε RNA+ and demonstrated that only 65% (60-70%) of Cε+ cells were Cε+ and Iε+, therefore a mean of 35% of Cε+ cells were Iε-. These results confirm that the two probes are equally sensitive as well as the presence of rearranged

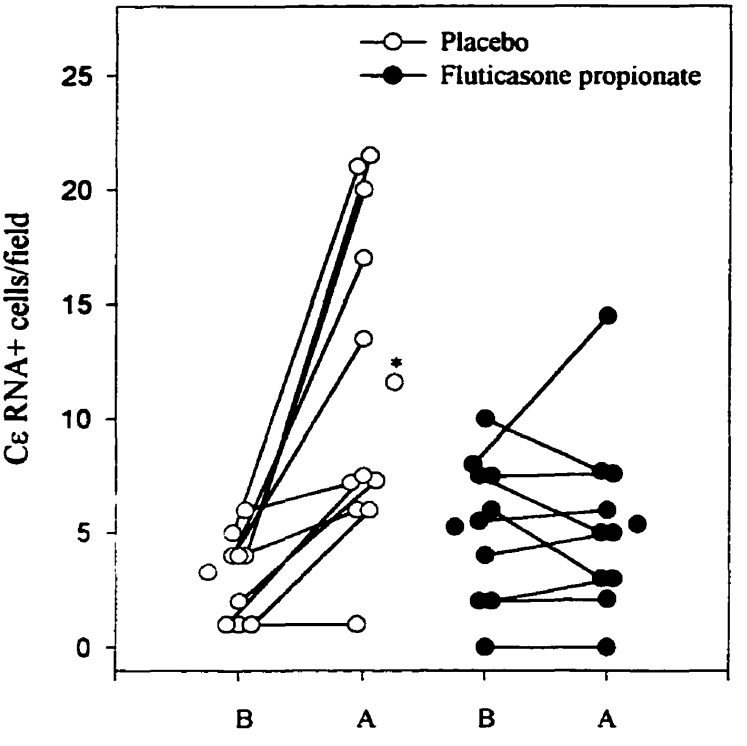


Figure 3 A. Expression of Cε RNA within the nasal mucosa of seasonal allergic rhinitis patients before (B) and after (A) *in vivo* allergen challenge. Placebo-treated patients (n=11) exhibited significantly more cells expressing Cε RNA following challenge, while there was no increase within tissue from patients pre-treated with FP (n=10; $p<0.01$).

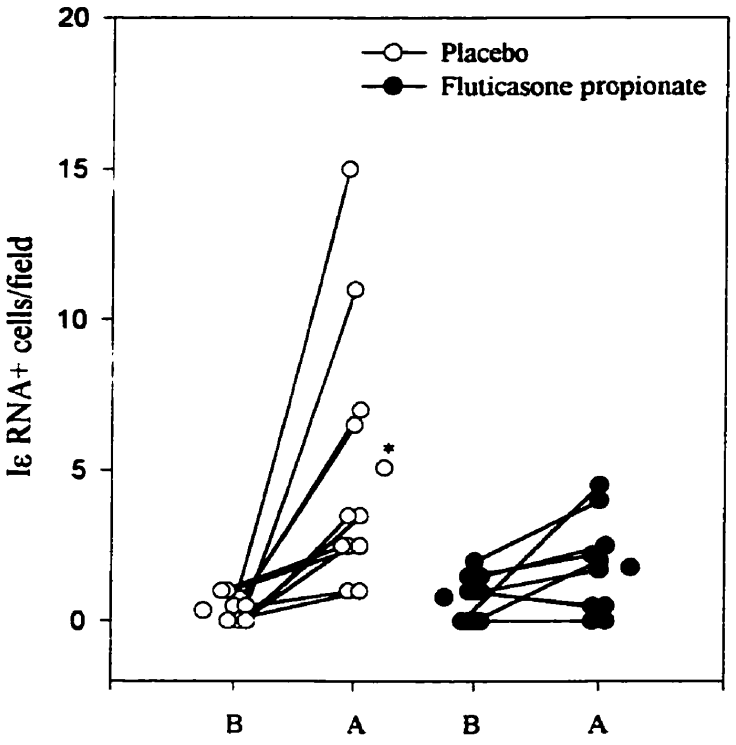


Figure 3 B. Expression of Iε RNA within the nasal mucosa of patients with seasonal allergic rhinitis before (B) and after (A) *in vivo* allergen challenge. There were significantly more cells expressing Iε RNA within tissue from patients treated with placebo (n=11; $p<0.01$), but not those that received FP (n=10), when pre- and post-challenge tissue was compared ($p>0.05$).

ε chain mRNA, Cε+/Iε- cells. Simultaneous immunocytochemistry and *in situ* hybridization confirmed that the Cε RNA+ cells were indeed CD20+ (Plate 3.1d)

In FP-treated patients, consistent with the observation that steroids inhibited the assessed clinical responses, there was no increase in the number of Cε RNA+ cells when pre- (5.3 ± 3.2) and post-challenge (5.4 ± 4.0, $p>0.05$) tissue was examined (Figure 3A). Although in some patients there appeared to be an increase in the number of Iε RNA+ cells following allergen challenge (0.8 ± 0.8), this was not statistically significant (1.8 ± 1.6, $p>0.05$; Figure 3B, Plate 3.1f). When the delta value of before *vs.* after allergen challenge was calculated for the number of cells expressing Cε or Iε RNA, the change observed within FP-treated patients was significantly less than patients that received placebo treatment ($p<0.05$; Figure 3A &B).

3.1d Expression of Cγ RNA after in vivo allergen challenge.

IgG has a number of subclasses which, except for IgG_4, are not highly expressed during an allergic reaction. A consensus oligonucleotide of a 30 bp sequence found in all 4 IgG subclasses was used as a negative control to confirm that observed increases in RNA product were specific responses. In contrast to the immunoglobulin ratio in the serum (IgG:IgE = 100,000:1) [Turner et al, 1996], the populations of Cγ RNA+ cells (3.2 ± 1.7) and Cε RNA+ cells (3.3 ± 1.6) in baseline biopsies of the nasal mucosa were similar. Allergen challenge resulted in a significant decrease in the number of CγRNA+ cells (1.6 ± 1.4), in contrast the observed increase in Cε and Iε RNA+ cells ($p<0.05$; Figure 3C), indicating the specific nature of the response to allergen. Furthermore, 6 weeks pre-treatment with FP inhibited this decrease, with the mean number of cells expressing CγRNA being highly similar within pre- (3.3 ± 1.4) and

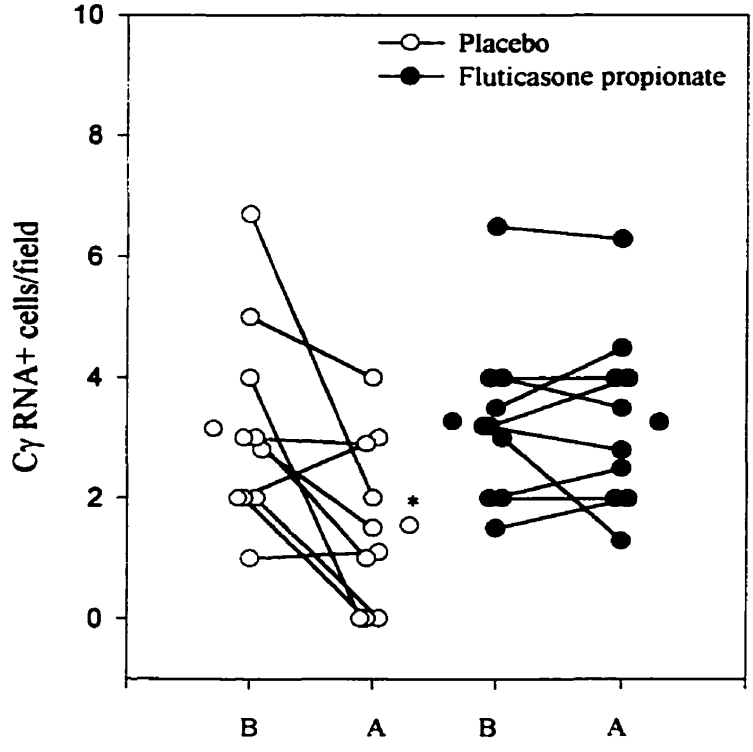


Figure 3 C. The number of cells expressing Cγ RNA transcripts within the nasal mucosa of seasonal allergic rhinitics patients before (B) and after (A) *in vivo* allergen challenge. Within tissue from placebo-treated patients (n=10) there was a significant reduction in the number of cells expressing Cγ RNA ($p<0.01$), while FP treatment (n=10) inhibited this change, when pre- and post-challenge tissue was compared ($p > 0.05$).

post-challenge tissue (3.3 ± 1.5, $p>0.05$; Figure 3C).

3.1e IL-4 mRNA after in vivo allergen challenge.

The presence of IL-4 mRNA+ cells within the nasal mucosa of seasonal allergic rhinitics has been previously demonstrated [Ying et al, 1994; Masuyama et al, 1994]. In light of its importance for ε germline transcription and isotype switching to IgE, however, it was useful to characterize its expression along with Cε and Iε RNA in the same patient group. There was a population of IL-4 mRNA+ cells within baseline biopsies (2.0 ± 2.3) from seasonal allergic rhinitis patients taken out of season. However, when pre- and post-challenge biopsies from placebo-treated patients were compared, an increase in the number of cells expressing IL-4 mRNA was observed (9.8 ± 6.7, $p<0.01$; Figure 4, Plate 3.1b). There was no change in the number of IL-4 mRNA+ cells in tissue obtained from patients that had received FP (4.1 ± 2.7 *vs.* 4.3 ± 3.4, $p>0.05$). The change, or delta value, in the number of cells expressing IL-4 mRNA within tissue from placebo-treated patients was significantly higher than those that received FP treatment ($p<0.05$; Figure 4). There was no correlation between the increase in IL-4 and Cε or the appearance of Iε RNA+ cells, most likely reflecting the fact that IL-4 expression occurs in different cell types, whereas the parallel increases in the numbers of Cε+ and Iε+ cells were due not only to production by the same cell type, but in many cases to identical cells.

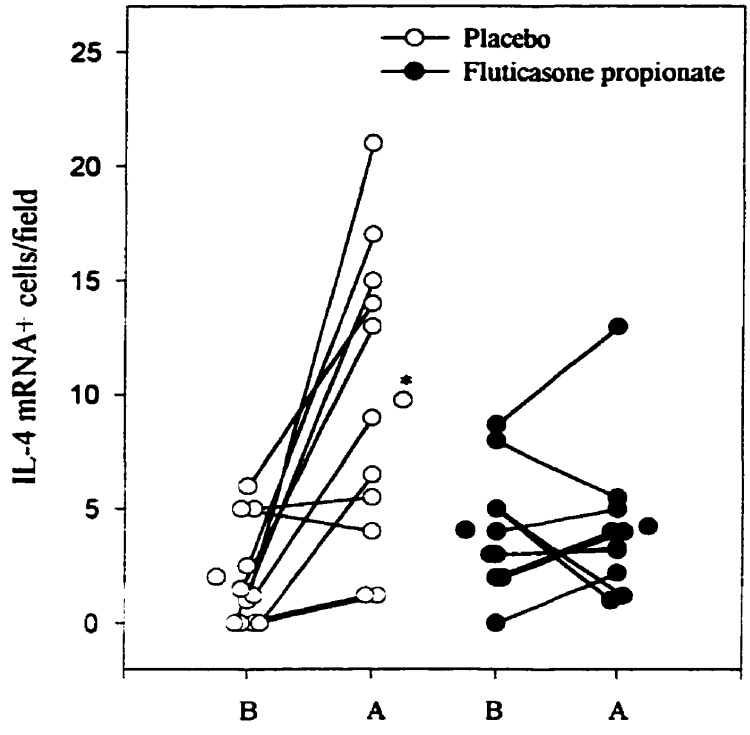


Figure 4. The expression IL-4 mRNA+ cells within the nasal mucosa of patients with seasonal allergic rhinitis before (B) and after (A) *in vivo* allergen challenge. There was a significant increase in the number of cells expressing IL-4 mRNA in placebo-treated patients (n=11; $p<0.01$), but not within tissue obtained from patients pre-treated with FP (n=10; $p>0.05$) when pre- and post-challenge tissue was compared.

the numbers of Cε+ and Iε+ cells were due not only to production by the same cell type, but in many cases to identical cells.

3.1f IgE protein after in vivo allergen challenge.

IgE protein-positive cells were more numerous in biopsy samples from seasonal allergic rhinitis patients outside the pollen season (12.6 ± 8.4), compared to normal control subjects (1.3 ± 2.4, $p<0.01$; Figure 5A). With double immunocytochemistry, a large percentage of the IgE+ cells were identified as mast cells (44%), while virtually no IgE protein was localized to CD20+ B cells (1%) or CD38+ plasma cells (1%; Figure 5B).

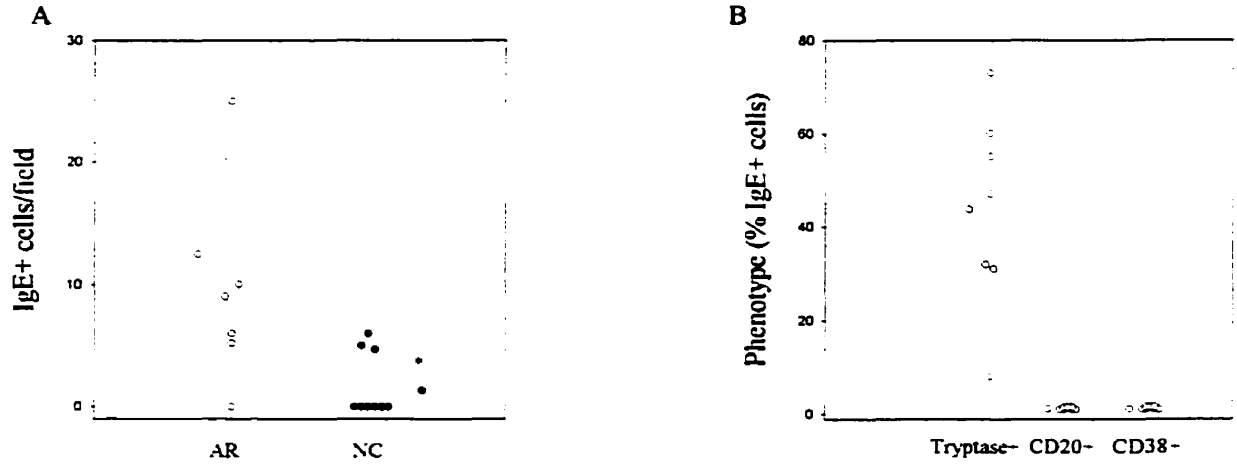


Figure 5. IgE protein within the nasal mucosa of patients with seasonal allergic rhinitis (AR) and normal controls (NC). There were more IgE-immunoreactive cells within allergic nasal tissue (n = 9) obtained out of season, compared to tissue from controls (A; [n = 9], $p<0.01$). The majority of this IgE appeared to be co-localized to the mast cell marker, tryptase (B; [n = 7], 43%).

3.2 Natural exposure to allergen

3.2a Clinical response after natural allergen exposure.

To confirm that changes observed in response to *in vivo* allergen challenge were representative of naturally occuring disease, the nasal mucosa of patients with seasonal allergic rhinitis was examined before and after allergen exposure during the pollen season. To avoid inter-group bias, the placebo- and FP-treated groups were well matched for age, gender and baseline nasal symptoms (Table 3.2). The patients' overall allergic response was assessed by weekly visual analogue scores (VAS). Taking the mean of the scores collected from each weekly visit, it appeared that patients who received a 6 week pre-treatment with FP experienced a marked reduction in seasonal symptoms with a mean VAS score of 12 (ranging from 5-30) and requirement for rescue medication compared with those of patients pre-treated with the matched placebo, 40 (30-50, p=0.003; Figure 6).

3.2b B cells within the nasal mucosa after natural allergen exposure.

CD20 immunoreactivity was observed within the nasal mucosa both prior to and during the pollen season. Similar to observations following *in vivo* allergen challenge, there was no significant increase in the number of these cells when pre- and post-season counts were compared for placebo- (4[3-8] *vs.* 4[1-7]) or FP- (3[1-6] *vs.* 5[1-9]; p>0.05) treated patients. Also in line with findings from allergen challenge, CD20 immunoreactive cells were predominantly clustered within the submucosa with some positive signal being detected in the epithelial cell layer.

Table 3.2 *Clinical characteristics of patients that underwent natural allergen exposure.*

	Treatment[a]	
	PL	**FP**
No. of patients	17	16
Age (yrs ± SD[b])	33.1 ± 10.7	32 ± 8.9
Gender (M:F)	12:5	10:6
Serum grass pollen-specific IgE (scale 0-5; mean ± SD)	3.2 ± 0.8	2.9 ± 0.8

[a]Patients received a six week pre-treatment with either placebo (PL) or fluticasone propionate (FP) nasal spray.
[b]Data is represented as the mean ± standard deviation (SD).

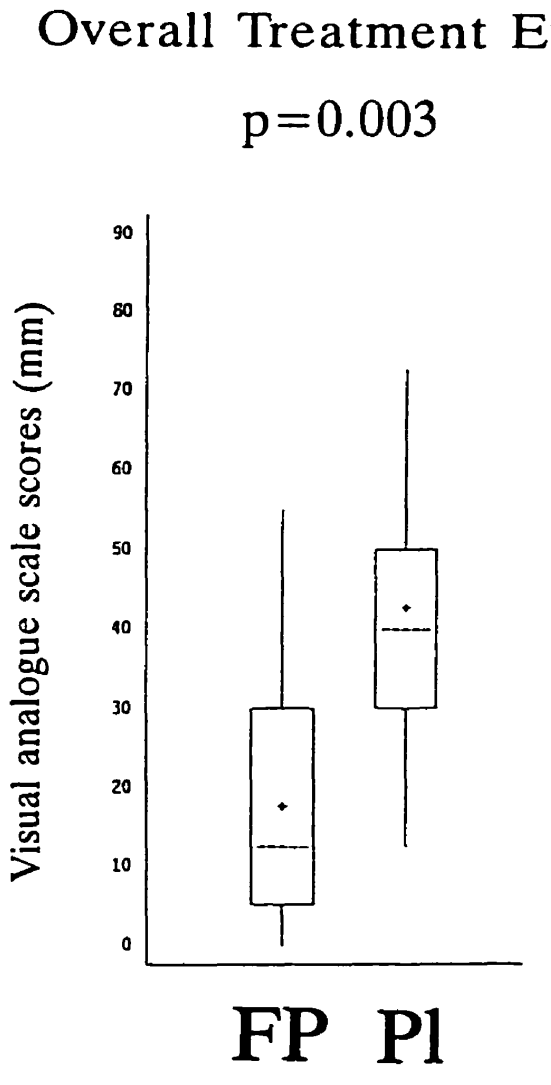


Figure 6. Clinical response as measured by visual analogue score (VAS). Following natural allergen exposure during the pollen season, patients pre-treated with fluticasone propionate exhibited significantly lower VAS scores than those who received a placebo treatment.

3.2c Expression of Cε and Iε RNA after natural allergen exposure.

Within sections of pre-season tissue obtained from patients that received placebo medication, there were a fair number of cells expressing Cε RNA (1.0 ± 0.7). When these counts were compared to the number within tissue obtained during the pollen season, however, there was an obvious and significant increase (3.6 ± 1.8, $p<0.001$; Figure 7A). While the majority of patients demonstrated no Iε RNA+ cells within nasal biopsy tissue obtained prior to the season (0.23 ± 0.34), during the season Iε RNA+ cells became clearly apparent (2.7 ± 1.5, $p<0.01$; Figure 7B, Plate 3.2a). In fact, there was over a 2 fold increase in pre *vs.* during season counts. As was observed following *in vivo* allergen challenge, there was a moderate correlation between the increase in the number of Cε RNA+ cells and the number of Iε RNA+ cells within nasal mucosal tissue obtained from patients that received placebo treatment ($r = 0.51$, $p<0.05$). Also similar to findings from allergen challenge, pre- and during season counts from FP-treated patients were variable. In some cases FP treatment reduced the number of Cε and Iε RNA+ cells compared to pre-season values, in some patients there was no change and in others FP treatment was associated with a persistent increase in Cε and Iε RNA+ cells. However, as a whole, patients pre-treated with FP did not exhibit increases in the number of Cε (1.3 ± 0.6 *vs.* 1.5 ± 1.2) or Iε (0.27 ± 0.38 *vs.* 0.53 ± 0.93; Plate 3.2b) RNA+ cells ($p>0.05$; Figure 7A, B). Furthermore, when the delta value was calculated for counts of before *vs.* after allergen exposure, analysis revealed that the rise in Cε and Iε RNA+ cells in was higher in placebo than FP - treated patients ($p<0.001$; Figure 7A, B).

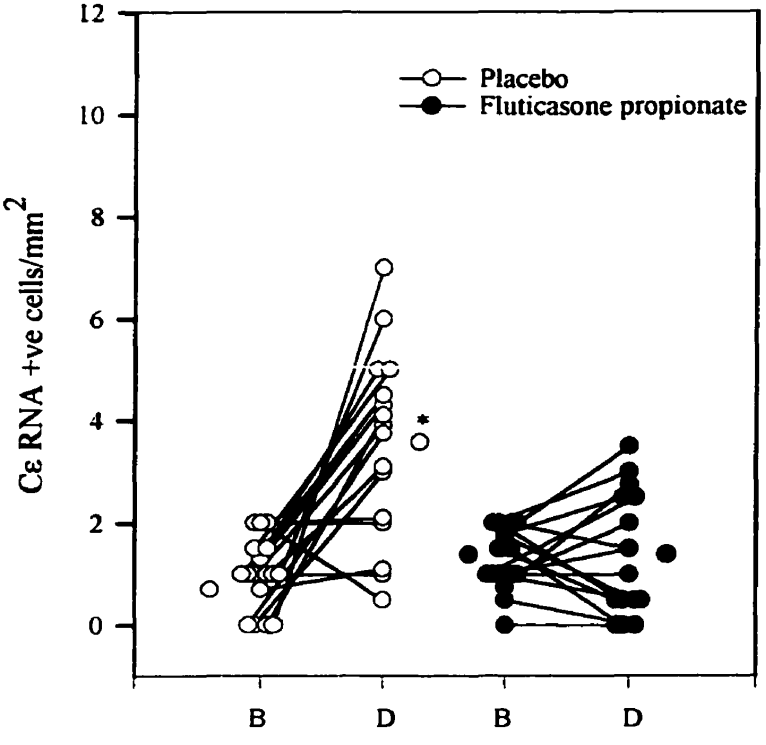


Figure 7 A. Expression of Cε RNA within the nasal mucosa of patients with seasonal allergic rhinitis before (B) and during (D) the pollen season. There were significantly more cells expressing Cε RNA within tissue from placebo-treated patients (n=17; $p<0.001$) following exposure to allergen, while FP-treatment inhibited this increase (n=16; $p>0.05$).

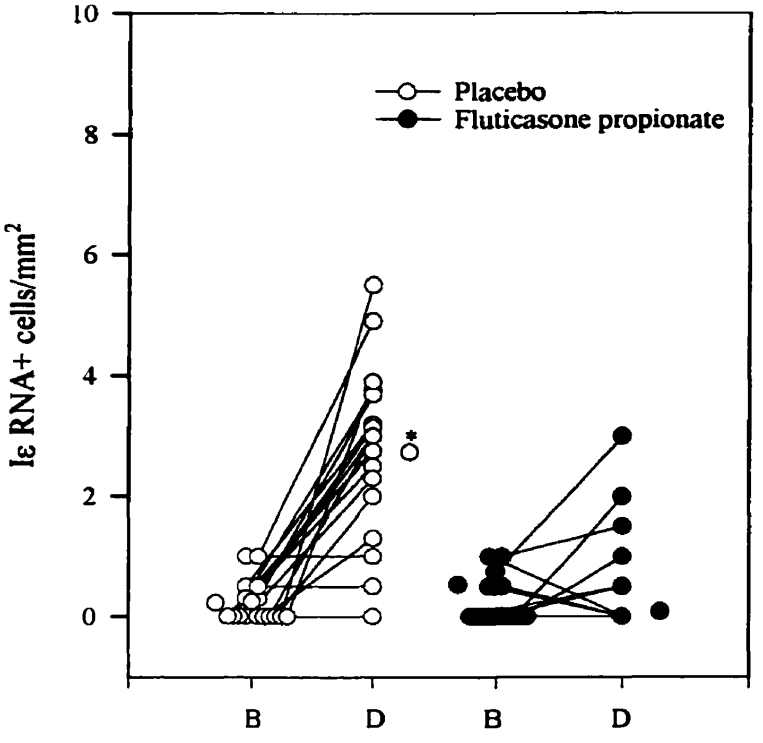


Figure 7 B. Expression of Iε RNA within the nasal mucosa of patients with seasonal allergic rhinitis before (B) and during (D) the pollen season. Tissue from patients treated with placebo (n=17) exhibited significantly more Iε RNA following exposure ($p<0.001$), while those that received FP (n=16) did not ($p<0.01$).

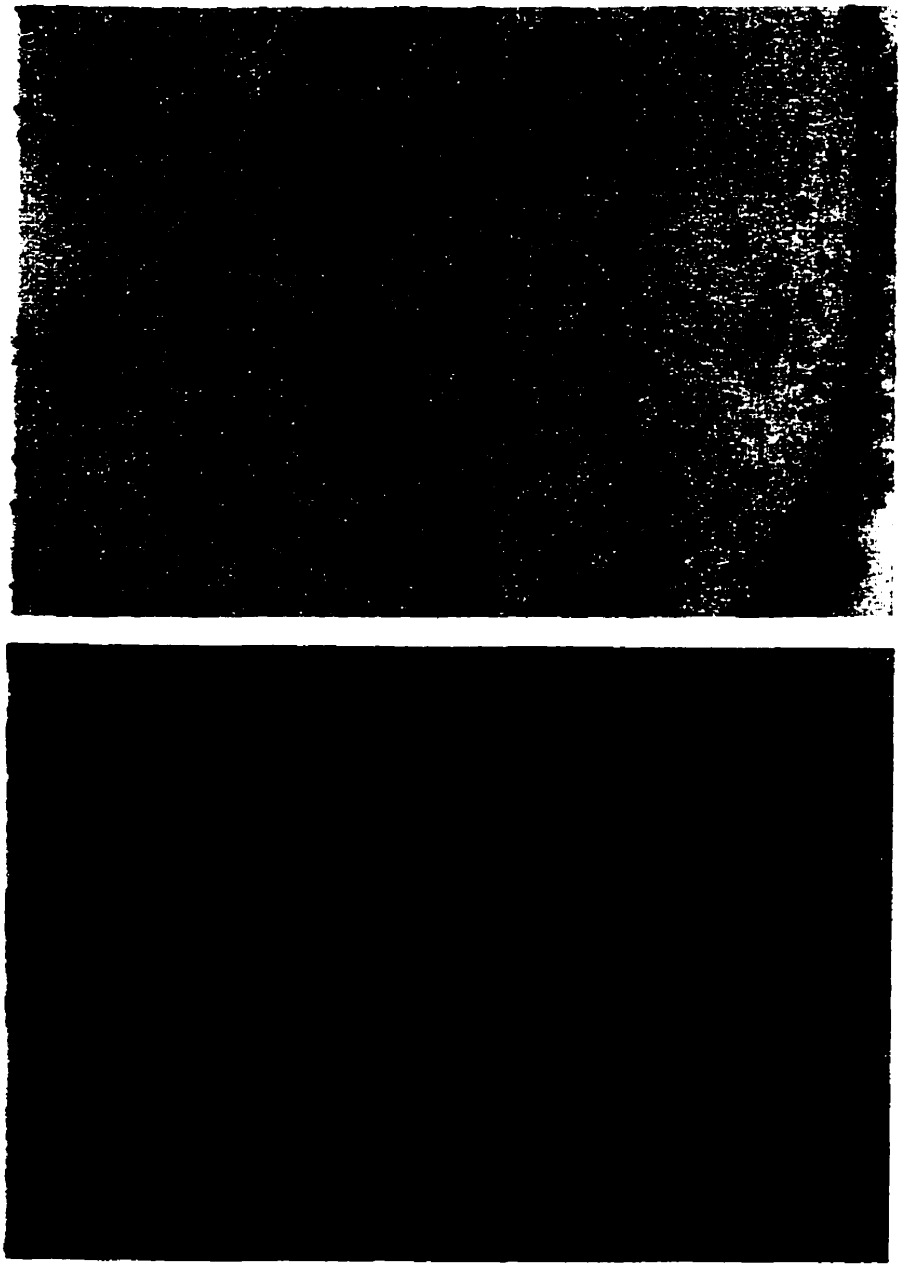


Plate 3.2 Representative photograph of *in situ* hybridization of cells expressing Iε RNA within sections of nasal tissue obtained from patients pre-treated with placebo (a) or fluticasone propionate (b).

3.2d Expression of Cγ RNA after natural allergen exposure.

The number of Cγ RNA+ cells were also examined within nasal mucosal tissue obtained before and during the pollen season. In constrast to the decrease observed following *in vivo* allergen challenge, there was no change in the number of cells expressing Cγ RNA within nasal tissue from placebo-treated patients when tissue obtained before (2.2 ± 1.9) and during (3.0 ± 1.9; $p>0.05$) the season was compared. Similar findings were observed when the number of Cγ RNA+ cells within the nasal mucosa of patients pre-treated with FP were examined (2.0 ± 1.6 *vs.* 3.0 ± 2.2; $p>0.05$, Figure 7C).

3.2e IL-4 mRNA after natural allergen exposure.

Although the presence of IL-4 has been observed within the nasal mucosa of individuals with perennial allergic rhinitis [Bradding et al, 1993; Pawankar et al, 1997] as well as those with seasonal allergic rhinitis following acute allergen challenge [Ying et al, 1994; Masuyama et al, 1994], these results are the first to demonstrate the presence IL-4 mRNA+ cells following natural exposure to allergen during the pollen season. In both groups, those that received placebo or FP, there were a number of cells expressing IL-4 mRNA prior to the season (2.1 ± 1.4, 1.9 ± 1.3). Placebo-treated patients exhibited a significant increase in the number of these cells within tissue obtained during the pollen season (5.4 ± 2.4, $p<0.01$), while patients pre-treated with FP did not display any change in the number of these cells when before (1.9 ± 1.3) and during (2.3 ± 1.4) season biopsies were compared (Figure 8). As was observed with Cε and Iε RNA+ cells, there was inter-patient variability in response to FP treatment (Figure 8). Unlike the allergen challenge

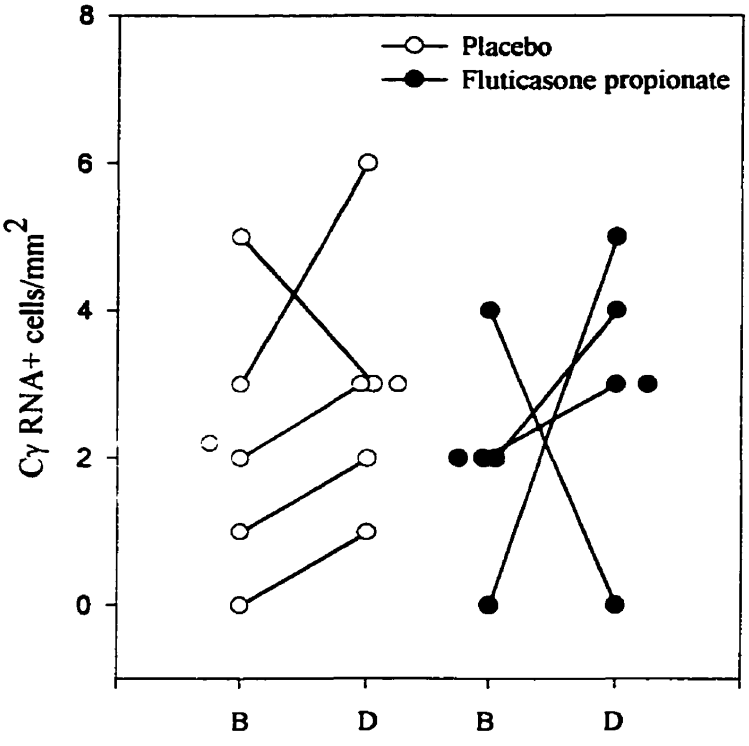


Figure 7 C. Expression of Cγ RNA+ cells within the nasal mucosa of patients with seasonal allergic rhinitis before (B) and during (D) the pollen season. There was no change in the number of cells expression Cγ RNA within tissue from either placebo (n=5) or FP-treated patients (n=4; $p>0.05$).

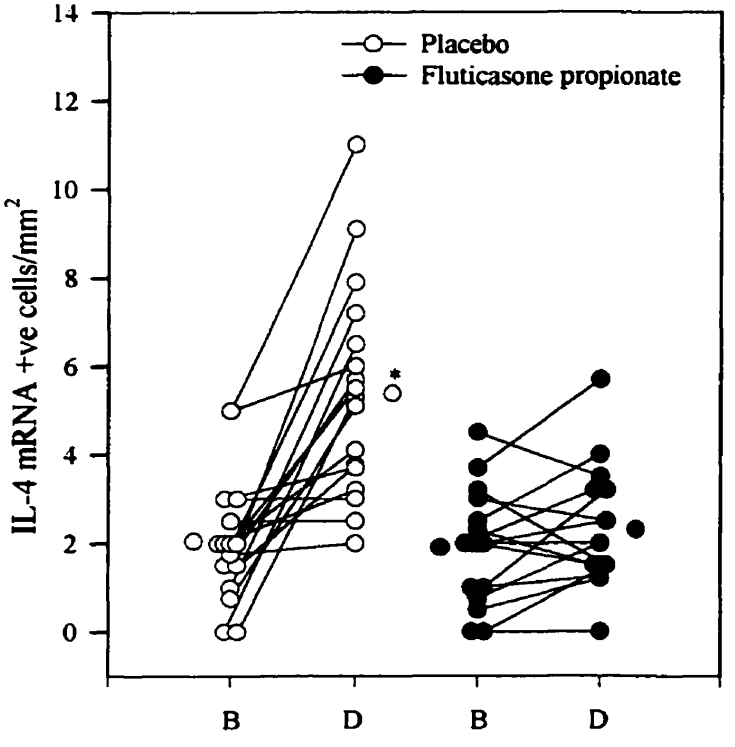


Figure 8. Expression of IL-4 mRNA+ cells within the nasal mucosa of patients with seasonal allergic rhinitis before (B) and during (D) the pollen season. A significant increase was observed following allergen exposure within tissue from patients pre-treated with placebo (n=17; $p<0.001$), but not FP (n=16; $p>0.05$).

data, however, there was a moderate correlation between the increase in the number of IL-4 mRNA+ cells and the appearance of Iε ($r = 0.58$, $p<0.05$).

3.2f Epsilon RNA within nasal mucosa and peripheral blood after natural allergen exposure.

In an attempt to characterize the relative amount of cells expressing ε RNA within the tissue compared to the blood, the number of Cε and Iε RNA+ cells were examined within nasal mucosa and peripheral blood of patients with seasonal allergic rhinitis obtained during the pollen season. Significantly more Cε and Iε RNA+ cells were observed within the tissue (5.5 ± 1.7, 3.0 ± 0.9) than the peripheral blood (3.1 ± 2.0, 1.1 ± 1.1/1000 PBMC) of the same patients. These results demonstrate that a proportion of circulating B cells that express Cε and Iε RNA. However, the fact that there are significantly more within the tissue would suggest that there is also local production of these RNA transcripts (Figure 9).

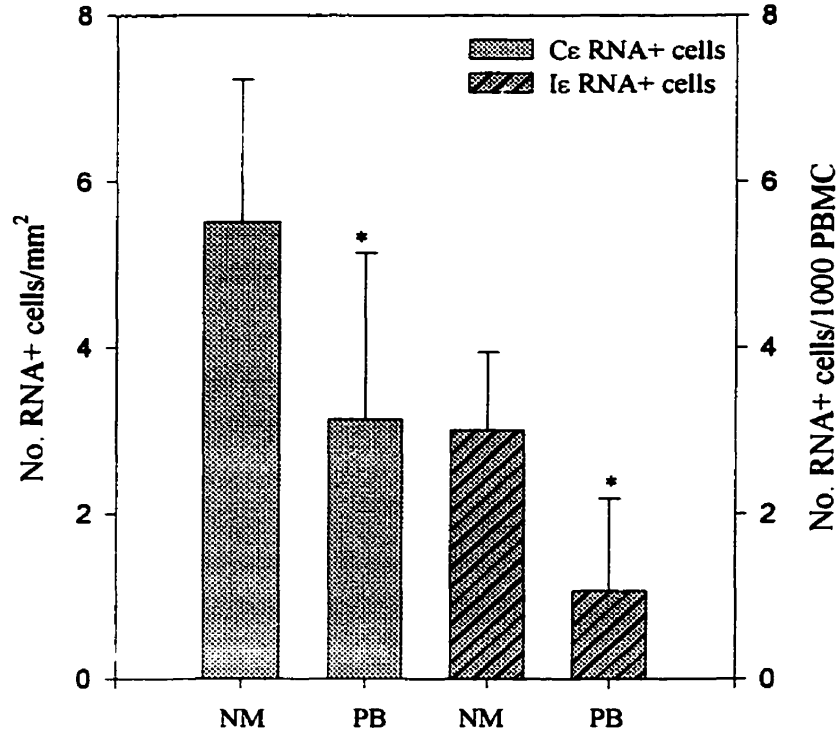


Figure 9. Comparison of Cε and Iε RNA within the nasal mucosa and peripheral blood of patients with seasonal allergic rhinitis. In both cases there were more cells expressing RNA transcripts of the ε gene within the nasal mucosa (NM; n=7) than the peripheral blood (PB; n=7, $p<0.01$).

3.3 *Ex vivo* antigen exposure

3.3a Nasal mucosal explant system

Results from *in vivo* allergen challenge and natural exposure experiments indicated that the hypothesized changes, the increases in Cε and Iε RNA+ cells, does indeed occur within the nasal mucosa of individuals with seasonal allergic rhinitis. To ensure that the observed changes were not merely due to cell infiltration, nasal mucosal tissue from patients with seasonal allergic rhinitis as well as non-allergic subjects (Table 3.3) was cultured with allergen-treated medium. Using this explant system, the possibility for cellular infiltration was eliminated and any alterations in RNA expression were therefore considered to be derived solely from the tissue constituents.

3.3b Proof of tissue integrity

Prior to employing this technique for analyzing questions regarding local ε germline transcription, close scrutiny of protocol and tissue intergrity were undertaken. Histological examination on hematoxylin and eosin stained sections determined that nasal mucosal tissue cultured for 24 hours in medium alone had normal tissue integrity and exhibited no apparent signs of cell death. Further assessment of inflammatory cell viability, whether they retain their cell surface markers, was also determined. With immunocytochemistry, an abundance of T cells (CD3+), a number of mast cells (tryptase +) and eosinophils (MBP+) as well as a small population of basophils (BB1+) were observed within nasal mucosa cultured with allergen for 24 hours (Plate 3.3a-d). These assessements indicated that the nasal mucosa is healthy after 24 hours culture and suitable for *ex vivo* study.

Table 3.3 *Characteristics of patients that donated nasal tissue for ex vivo allergen exposure*

	Allergic	Non-allergic
No. of patients	14	9
Age (yrs ± SD[a])	38.6 ± 13.2	51.3 ± 13.6
Gender (M:F)	7:6	5:4
Skin test to ragweed	+	-

[a]Data represented as the mean ± standard deviation (SD).

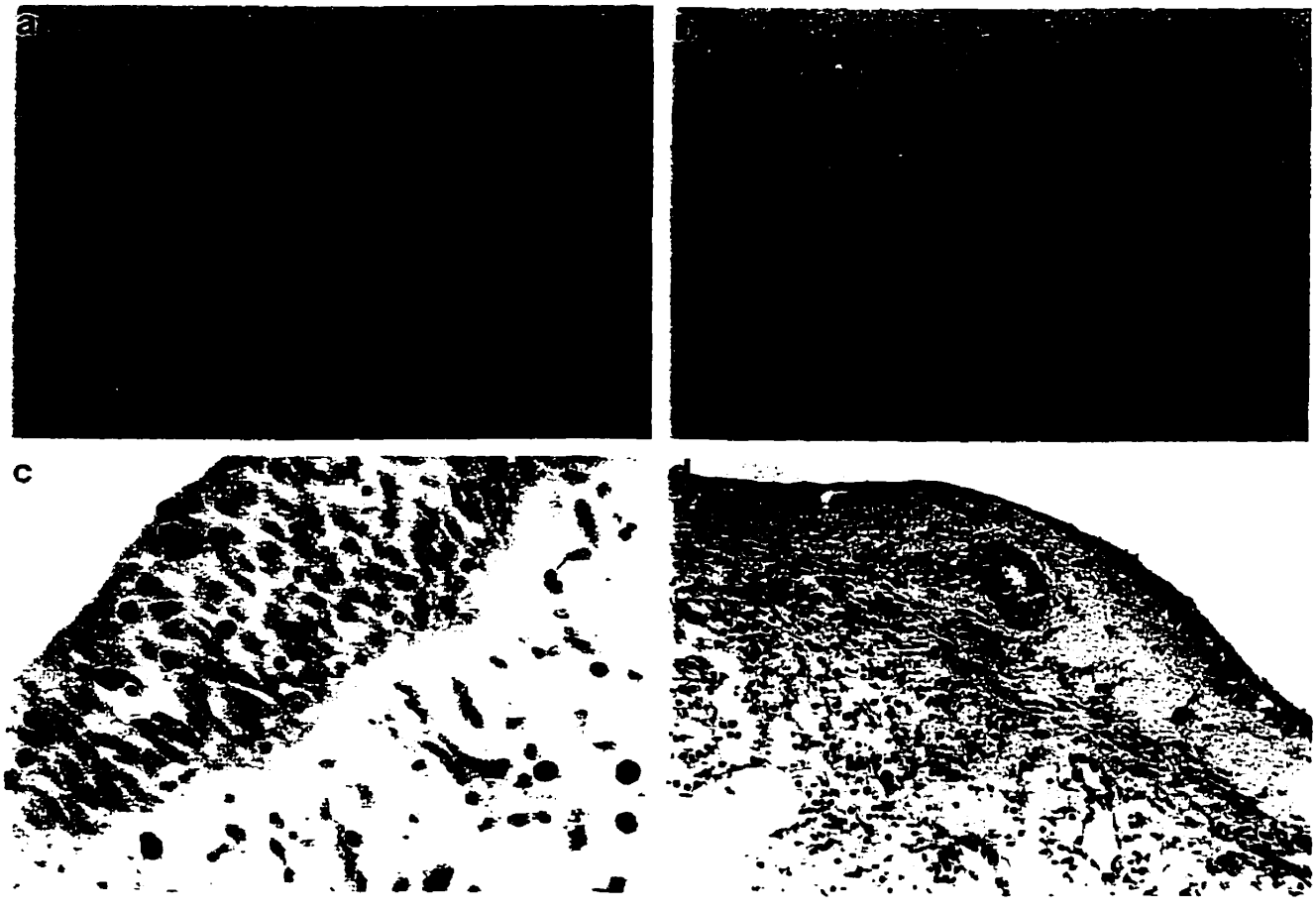


Plate 3.3 Integrity of nasal mucosal tissue after 24 hours of culture. Immunocytochemistry using antibodies directed against proteins that identify (a) T cells (CD3), (b) mast cells (tryptase), (c) eosinophils (major basic protein) and (d) basophils (BB1) demonstrates the viable state of these cells.

3.3c Response of nasal mucosal tissue to ex vivo allergen challenge.

Although previous work has demonstrated no difference in symptom scores when doses of 10-1000 PNU of ragweed allergen were used in nasal provocation studies [Doyle et al, 1995], the conditions for *ex vivo* challenge of nasal mucosal tissue with ragweed allergen had not been determined. We therefore performed a dose response with 5, 50, 500 and 1000 PNU/ml of ragweed allergen (n=4). The number of IL-4 mRNA+ cells in sections of nasal mucosal tissue cultured for 24 hours with the allergen diluent did not change compared to the number within tissue cultured within medium alone. Culturing the tissue with increasing concentrations of ragweed allergen, 5 PNU to 500 PNU/ml, resulted in a progressive increase in the number of IL-4 mRNA+ cells, however, no further elevation was noted at 1000 PNU. Experiments for this study were therefore carried out with 500 PNU/ml of ragweed allergen (Figure 10).

3.3d B cells within allergic and non-allergic tissue after ex vivo allergen challenge.

CD20 immunoreactive cells were dispersed throughout the submucosa of nasal biopsies obtained from both subject groups. There were significantly more B cells within allergic (10.5[3-22]) compared to non-allergic (5[2-7]) tissue cultured in medium alone ($p<0.05$). No increase was observed in their number after *ex vivo* exposure to allergen (12.8[4-19], 4[1-7]; $p>0.05$). Within allergic nasal tissue, however, the B cells did seem to relocate following exposure to antigen, as they were observed mainly just beneath (Plate 3.4a) or infiltrating the epithelial layer (Plate 3.4b). Furthermore, they were often found in clusters, as was observed following *in vivo* allergen exposure (Plate 3.4c).

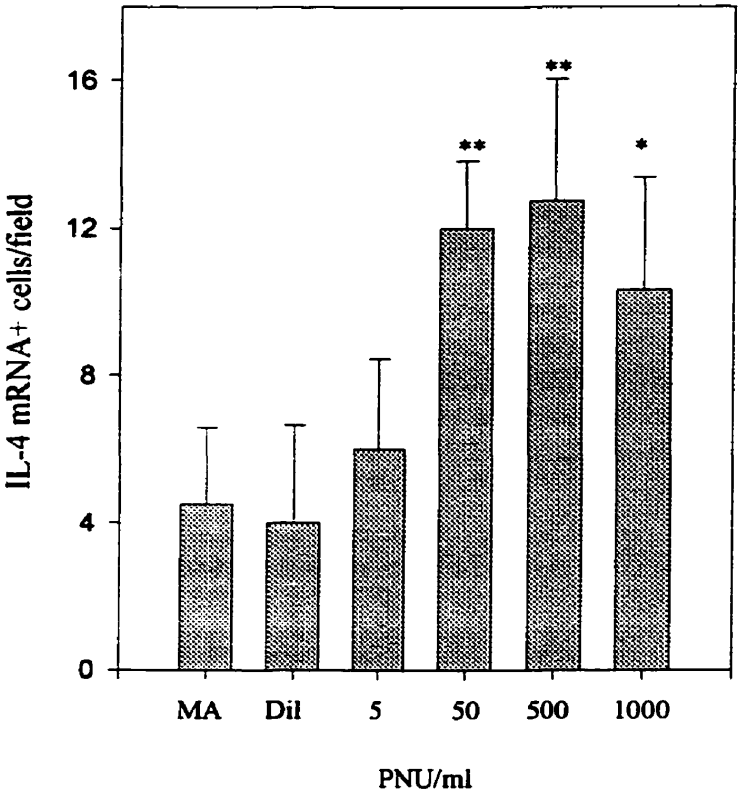


Figure 10. Expression of IL-4 mRNA in response to allergen concentration. There was a progressive increase in the number of cells expressing IL-4 mRNA when tissue was cultured in doses of 5-1000 PNU of allergen/ml. There was no significant affect when tissue was cultured with the allergen diluent (Dil) compared to medium alone (n=4; ** $p<0.01$, * $p<0.05$ by Dunnett's test for comparison of multiple conditions to one control).

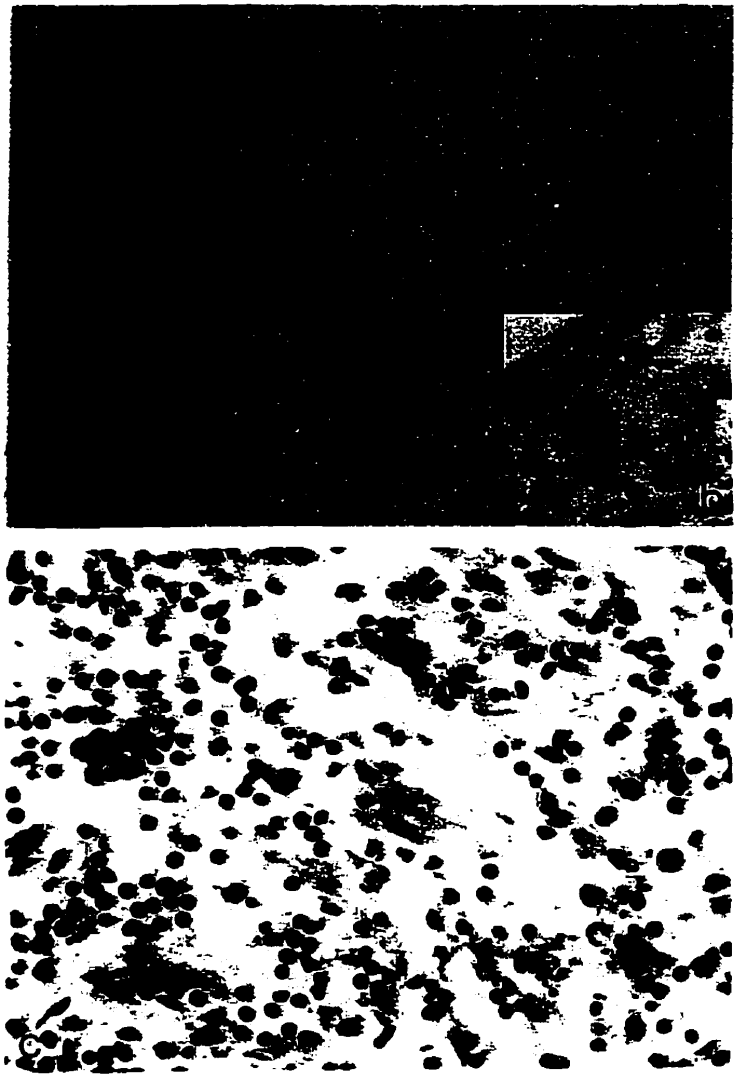


Plate 3.4 B lymphocytes identified on the basis of CD20 immunoreactivity within allergic nasal mucosa following 24 hours culture Note that they were observed just beneath the basement membrane (a), to infiltrate the epithelial layer (b) and in clusters of three or four cells within the submucosa (c).

3.3e Expression of Cε and Iε RNA after ex vivo allergen challenge.

In nasal tissue from patients with allergic rhinitis as well as non-allergic controls some cells expressing Cε RNA following culture in medium alone (2.8 ± 1.5, 1.5 ± 1.2, respectively) were observed (Figure 11A, Plate 3.5a). On the other hand, there were very few Iε RNA+ cells within sections of unstimulated allergic (0.42 ± 0.8) and non-allergic (0.33 ± 0.5; Figure 11B) tissue. Within nasal mucosal tissue cultured with allergen-treated medium, there was a significant increase in the number of Cε RNA+ cells (9.6 ± 3.3, $p<0.05$; Figure 11A, Plate 3.5b). Furthermore, Iε RNA+ cells became detectable within tissue that had otherwise been negative and was increased within tissue that had exhibited baseline numbers of Iε RNA+ cells (5.7 ± 1.8, $p<0.05$; Figure 10B, Plate 3.5c). Using simultaneous immunocytochemistry and *in situ* hybridization it was determined that 37% of CD20+ cells were Cε RNA+, while 32% were Iε RNA+, indicating that a considerable proportion of B cells within the tissue appear to be committed to IgE synthesis. Within tissue obtained from non-allergic patients there were no significant increases in either the number of Cε or Iε RNA+ cells when stimulated (1.8 ± 1.5, 0.44 ± 0.7) and unstimulated (1.6 ± 1.2, 0.33 ± 0.5; $p>0.05$) tissue was compared, although close examination of the data does show that in some cases there were increases (Figure 11A, B).

3.3f Expression of Cγ RNA after ex vivo allergen challenge.

To confirm that the increase in the number of Cε RNA+ cells was a specific response to allergen, sections of nasal tissue from allergics were also hybridized with anti-sense probes for Cγ RNA. Unlike the significant increase in Cε RNA+ cells observed following culture

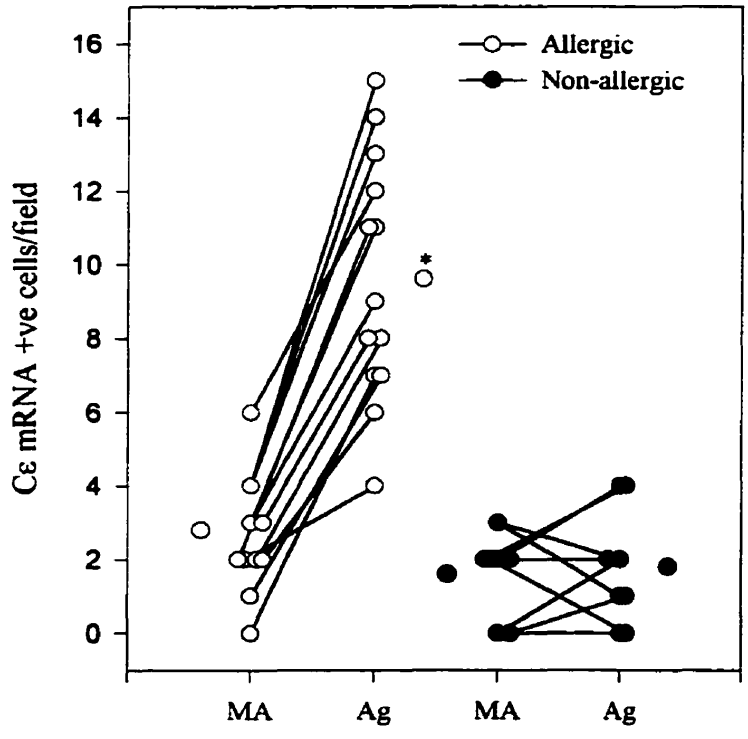


Figure 11 A. Expression of Cε RNA within nasal mucosal explant tissue cultured in specific allergen (Ag) or media alone (MA). There were significantly more cells expressing Cε RNA within allergic (n=13; $p<0.001$), but not non-allergic (n=9; $p>0.05$), tissue cultured with allergen compared to medium alone.

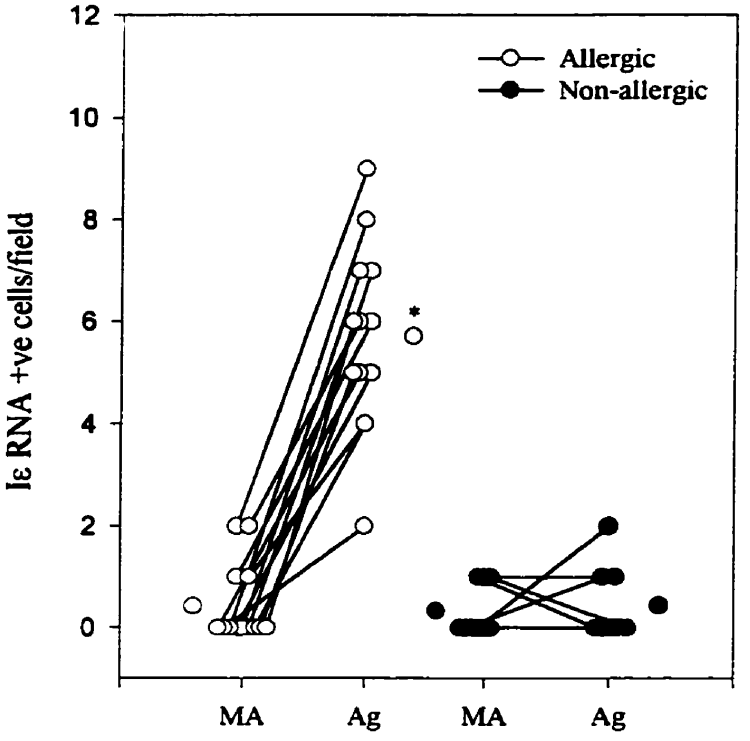


Figure 11 B. Expression of Iε RNA within nasal mucosal explant tissue following culture with specific allergen (Ag) or medium alone (MA). Significantly more cells expressing Iε RNA were observed within allergic nasal tissue cultured with allergen (n=13; $p<0.01$), compared to medium alone, while increases were not observed within nasal tissue obtained from non-allergic patients (n=9; $p>0.05$).

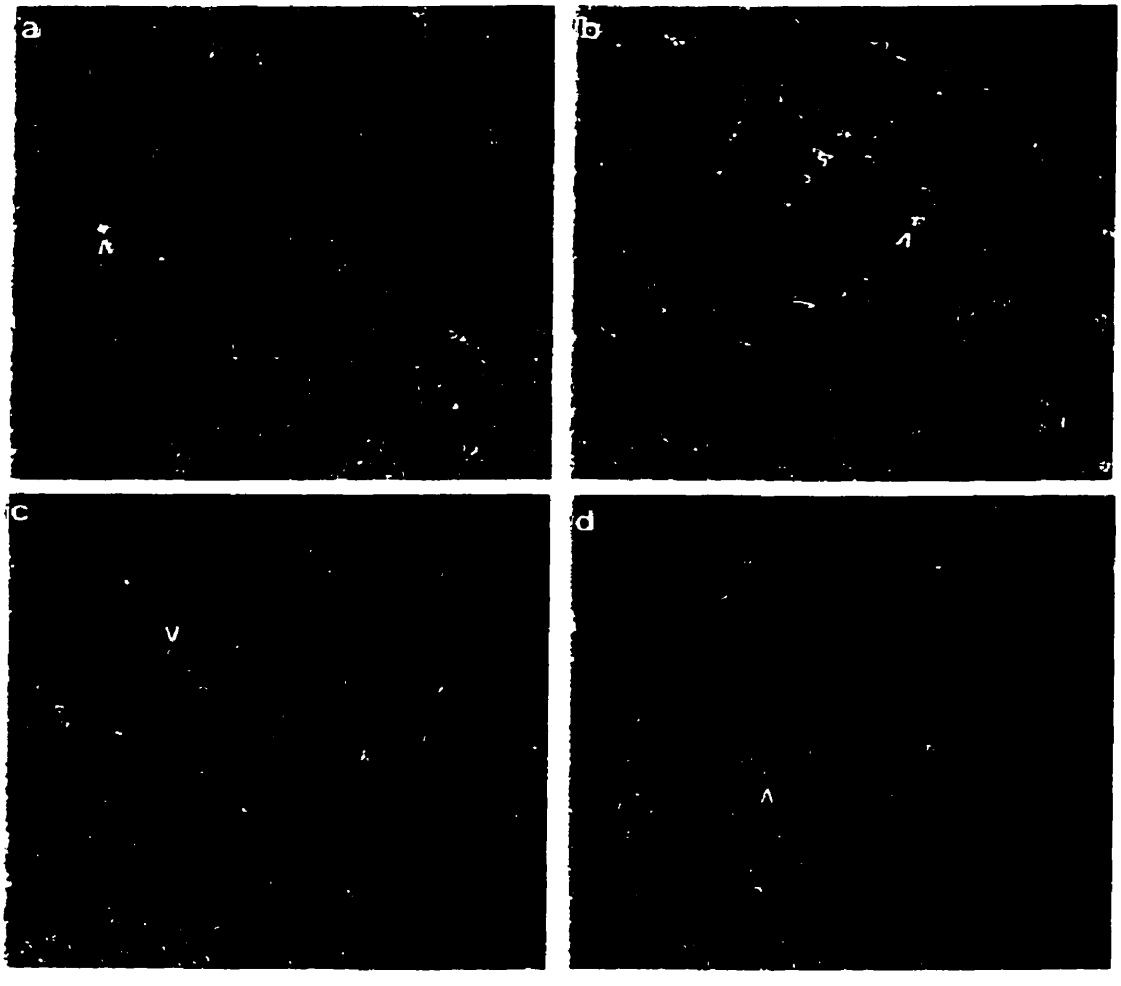


Plate 3.5 Representative photographs of RNA expression in cultured nasal mucosal tissue using *in situ* hybridization, as visualized under darkfield illumination. There were a small number of Cε RNA+ cells within nasal tissue cultured in medium alone (a), while a significant number of cells expressing RNA encoding Cε (b) and Iε (c) transcripts was observed in stimulated tissue. Similarly, a substantial number of IL-4 mRNA+ cells was observed in tissue cultured in allergen-treated medium (d).

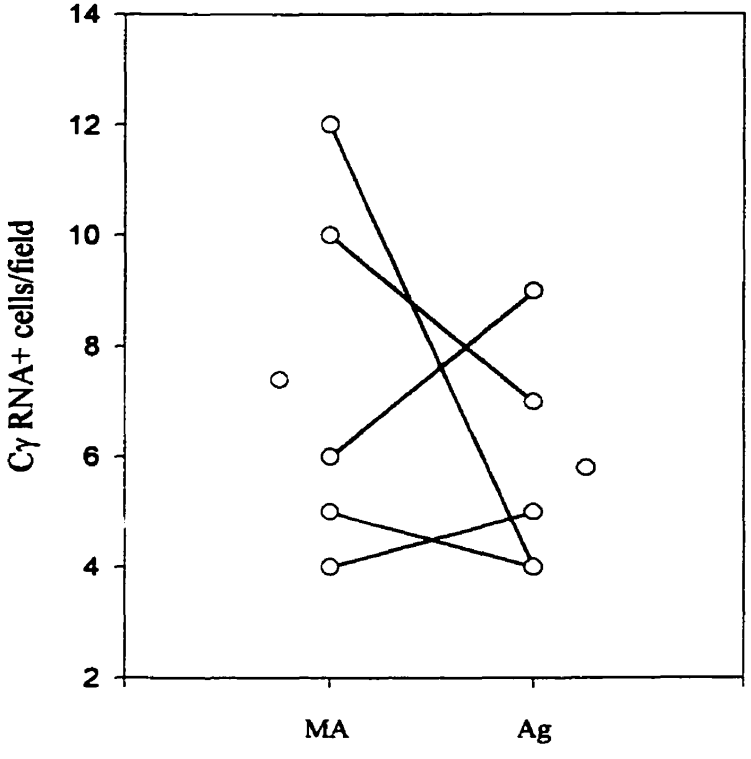


Figure 12. Expression of Cγ RNA within nasal mucosal explant tissue obtained from patients with seasonal allergic rhinitis. There was no significant difference between the number of Cγ RNA+ cells within tissue cultured with specific allergen (Ag) compared to medium alone (MA; n=5, $p>0.05$).

of allergic tissue with allergen, there was no change in the number of Cγ RNA+ cells within stimulated (5.8 ± 2.2) compared to unstimulated (7.4 ± 3.4; $p>0.05$, Figure 12) tissue.

3.3g IL-4 and IL-13 mRNA after ex vivo allergen challenge.

In light of the fact that IL-13 as well as IL-4 induce ε germline transcription and isotype switching to IgE, the expression of both cytokines was examined in this *ex vivo* system. There were a number of IL-4 mRNA+ cells within sections of unstimulated tissue from both allergic (4.2 ± 2.6) and non-allergic (2.0 ± 1.5; Figure 13A) patients. Although there were two-fold more IL-4 mRNA+ cells within allergic tissue, this difference did not reach significance ($p>0.05$). IL-13 mRNA+ cells were similarly represented in both tissue types following culture in medium alone (6.6 ± 3.0, 5.3 ± 2.3; $p>0.05$; Figure 13B), while there were more IL-13 than IL-4 mRNA+ cells within sections of nasal mucosa from non-allergic patients ($p<0.01$; Figure 11). Significantly higher numbers of cells expressing mRNA for IL-4 (10.3 ± 3.5; Plate 3.5d) and IL-13 (13.4 ± 7.2) were observed within allergic nasal tissue cultured with allergen-treated medium compared to medium alone ($p<0.01$; Figure 13A&B). Furthermore, simultaneous immunocytochemistry and *in situ* hybridization demonstrated that the majority of cells expressing IL-4 mRNA were CD3+ cells (70%) and tryptase+ cells (32%). While IL-13 mRNA was also co-localized to CD3+ (44%) and tryptase+ (18.5%) cells, it was substantially less than IL-4, findings consistent with previous work demonstrating that the macrophage is also a potent source of IL-13 [Hancock et al, 1998]. Interestingly, it appears that only a modest proportion of CD3+ and tryptase+ cells were expressing

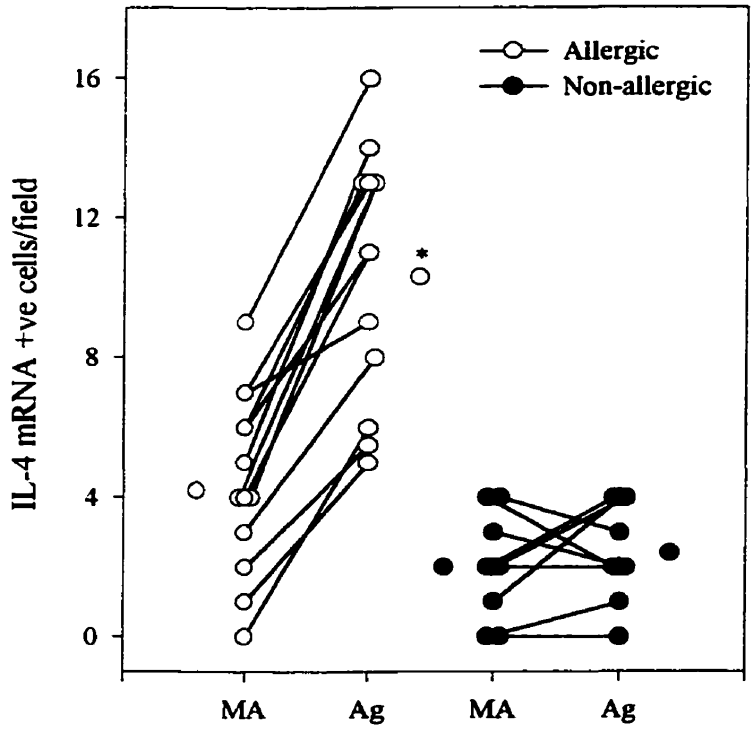


Figure 13 A. Expression of IL-4 mRNA+ cells within nasal mucosal explant tissue cultured with specific allergen (Ag) or medium alone (MA). The number of cells expressing IL-4 mRNA was significantly increased within tissue obtained from patients with seasonal allergic rhinitis (n=13) cultured with allergen ($p<0.001$), but not within tissue from non-allergic patients (n=9; $p>0.05$).

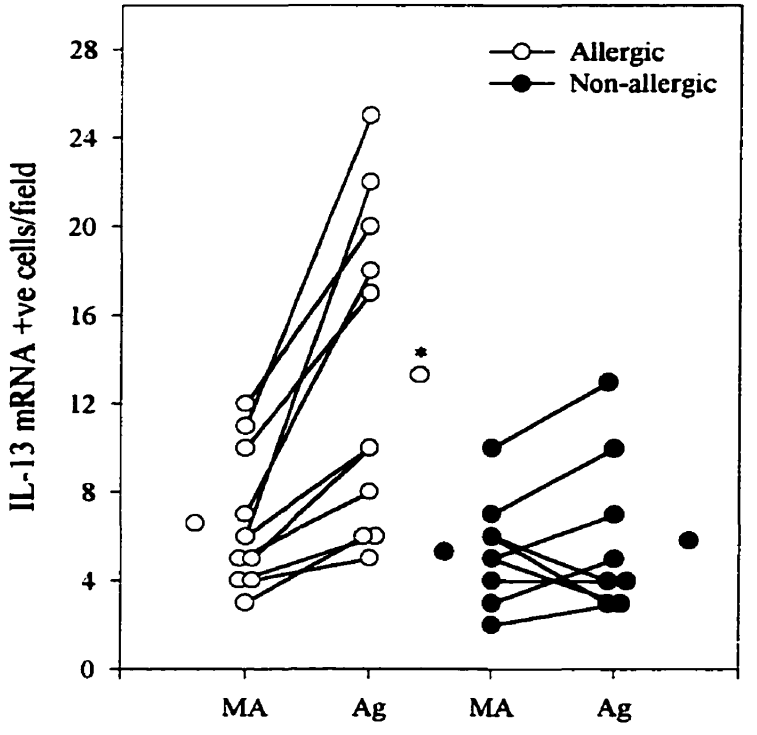


Figure 13 B. Expression of IL-13 mRNA+ cells within nasal mucosal explant tissue cultured with specific allergen (Ag) or medium alone (MA). Significantly more cells expressing IL-13 mRNA were observed within tissue from patients with seasonal allergic rhinitis (n=11) following culture with allergen than medium alone ($p<0.01$), while no increases were observed within tissue from non-allergic patients (n=9; $p>0.05$).

Table 3.4	*Co-localization of IL-4 and IL-13 mRNA*[a]		*T cell and mast cell production of IL-4 and IL-13 mRNA*[b]
IL-4/CD3	70% (62.5 - 80)[c]	**CD3/IL-4**	11% (10.2 - 11.8)
IL-4/Tryptase	32% (22.2 - 40)	**Tryptase/IL-4**	13.2% (10.3 - 21.1)
IL-13/CD3	44% (42.8 - 43.8)	**CD3/IL-13**	11% (8.5 - 12.5)
IL-13/Tryptase	18.5% (16.7 - 20)	**Tryptase/IL-13**	10% (7.1 - 12)

[a]Percentage of IL-4 and IL-13 mRNA+ cells that co-localize to T cells (CD3) and mast cells (tryptase).
[b]Percentage of T cells and mast cells that produce IL-4 and IL-13 mRNA.
[c]Data is represented as the mean % (range).

mRNA coding for IL-4 (11%,13%) and IL-13 (11%,10%) within allergen-stimulated tissue (Table 3.4). Within nasal tissue from non-allergic patients, there was no significant increase in the number of cells expressing IL-4 and IL-13 mRNA when stimulated (2.4 ± 1.4, 5.8 ± 3.6) and unstimulated tissue (2.0 ± 1.5, 5.3 ± 2.3; $p>0.05$; Figure 13A, B) was compared.

3.4 Comparison of the three exposure protocols.

When the change in the number of cells expressing Cε, Iε and IL-4 RNA in each allergen exposure protocol was examined, *i.e.* when the delta values (Δ; after - before) were calculated and compared, no significant differences were observed (Figure 14). However, when a comparison was made across the three protocols of allergen exposure, it was observed that the Δ value for each transcript was significantly less following natural allergen exposure than allergen challenge, either *in vivo* or *ex vivo* (Figure 15).

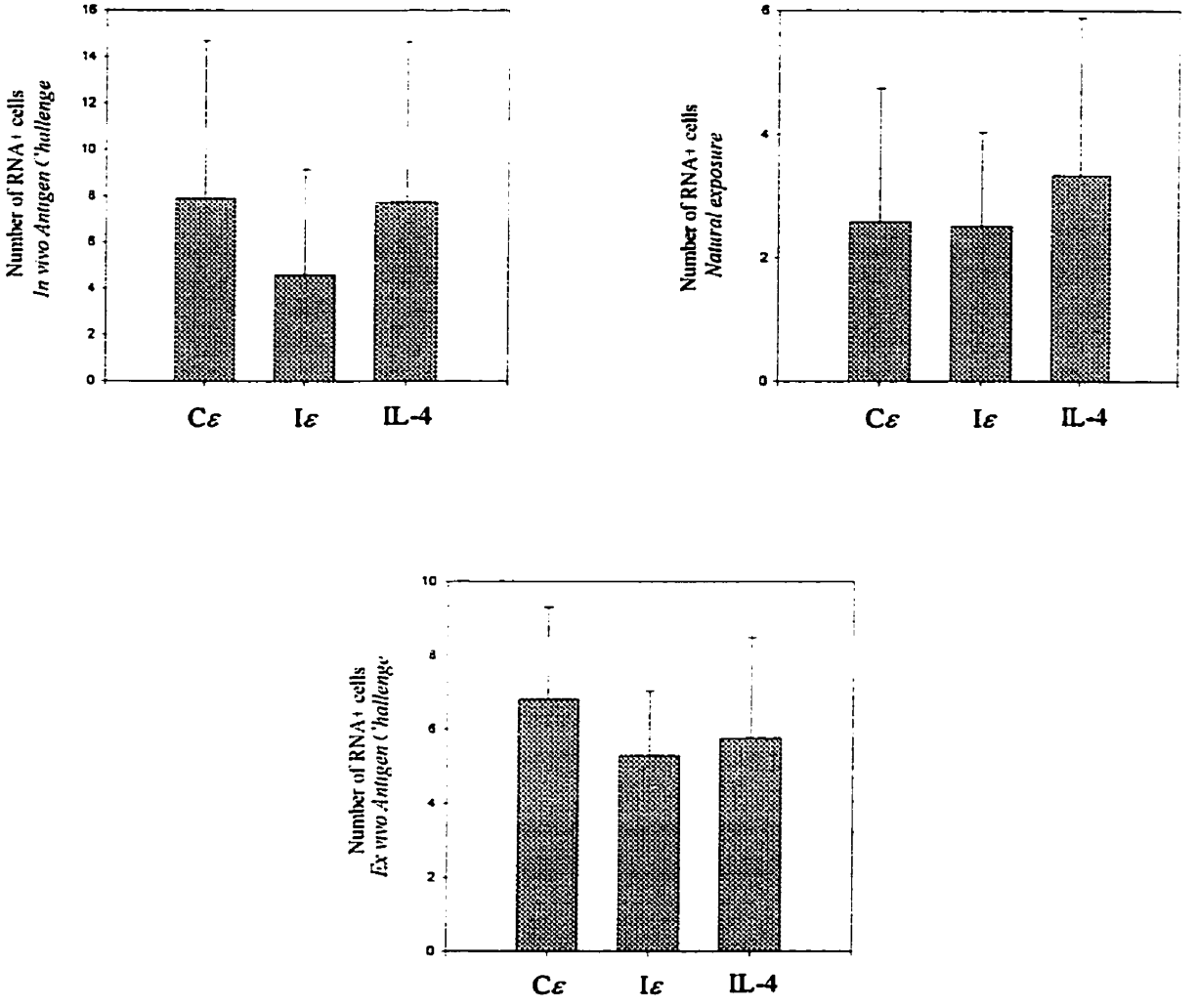


Figure 14. The change [after - before] in the expression of Cε, Iε and IL-4 RNA+ cells in nasal mucosal tissue from patients exposed to allergen by *in vivo* challenge (A), natural exposure (B), or *ex vivo* challenge (C). The increase in the number of cells expressing these RNA transcripts were similar.

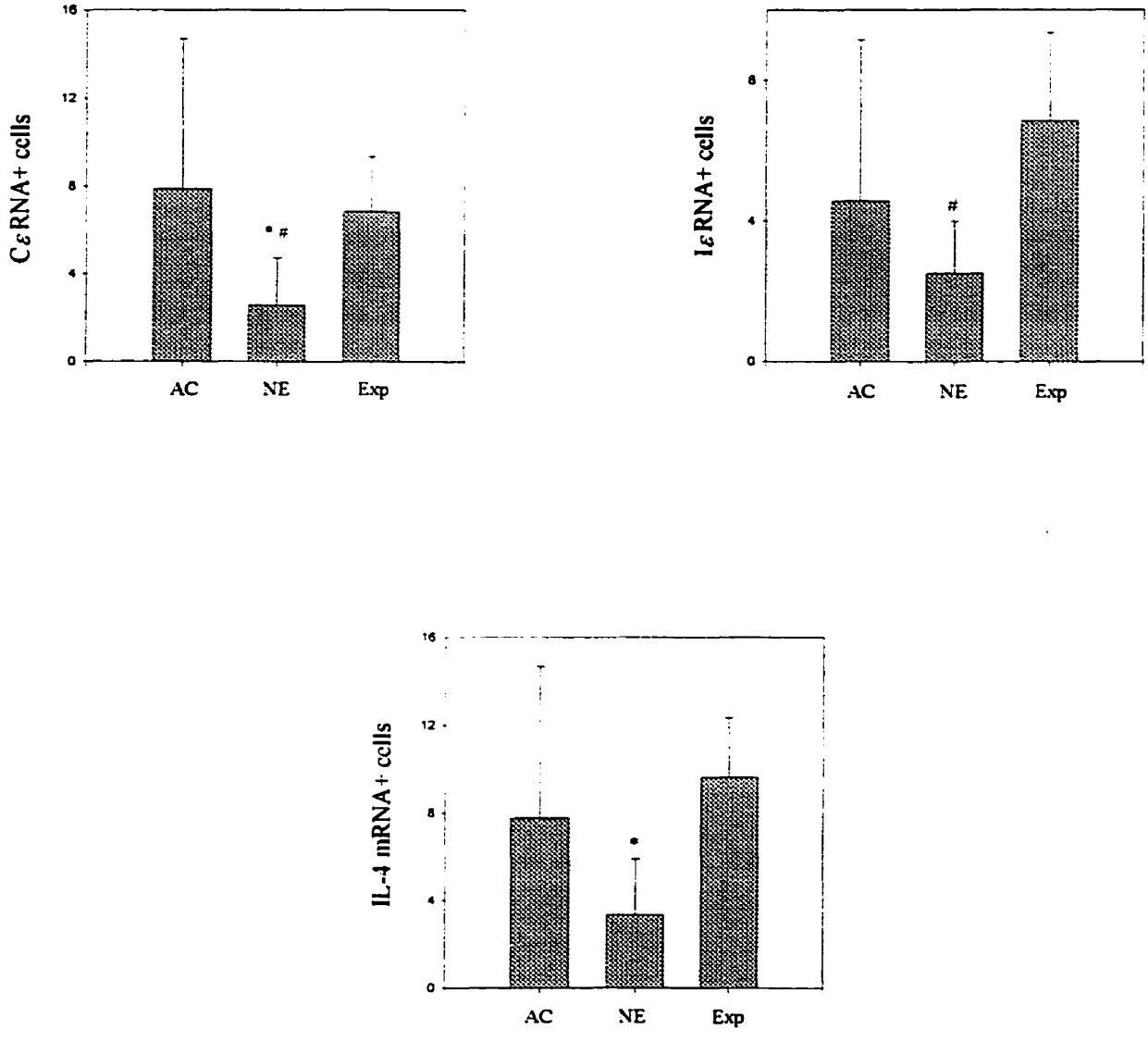


Figure 15. Comparison of the delta values [before - after] in expression of Cε, Iε and IL-4 RNA+ cells when the *in vivo* antigen challenge (AC), natural exposure (NE) and *ex vivo* allergen challenge (Exp) protocols was compared.
$^{*}p < 0.05$, AC *vs.* NE; $^{\#}p < 0.05$, NE *vs.* Exp.

'The unexamined life is not worth living.'
- Socrates

Chapter 4

4.0 DISCUSSION

4.1 Introductory remarks

Medical research is an endeavor to understand the mechanisms by which the human body functions as well as *mal*-functions. In 1968, IgE was recognized as the 'reaginic antibody' involved in allergic disease [Bennich et al, 1968] and subsequently detected in nasal secretions as well as nasal tissue of individuals with seasonal allergic rhinitis [Merrett et al, 1976; Ohashi et al, 1987]. While these reports illustrated the presence of IgE within this compartment, they did not establish the site from which it originated. The provenance of this Ig within the nasal mucosa has been mainly attributed to infiltrating IgE-bound inflammatory cells, IgE-producing plasma cells or to free IgE from the serum, however, a further possibility is the *in situ* production of IgE by resident B lymphocytes. The latter has been suspected for some time, based on reports of patients with negative skin reactions and RAST measurements, but allergen-specific IgE in nasal secretions [Huggins et al, 1975]. Furthermore, some patients with seasonal allergic rhinitis have also been observed to have specific IgE in nasal but not salivary secrections [Platts-Mills et al, 1979]. These findings set the foundation for the present work.

This thesis provides evidence for B cell commitment to IgE production, an event previously considered to take place exclusively in lymphoid organs, within the nasal mucosa of individuals with seasonal allergic rhinitis. The results shown here were acquired using nasal mucosal tissue obtained from patients that received either an *in vivo* allergen challenge or natural allergen exposure during the pollen season. It was also demonstrated that pre-treatment with intra-nasal corticosteroid inhibited B cell commitment to IgE as well as synthesis of the IgE-switch factor, IL-4. The third study of this trilogy examined the effects of exposing allergic nasal mucosal tissue to allergen *ex vivo,* by culturing the tissue in allergen-treated medium. This explant system provided a means to isolate the nasal tissue from systemic circulation and to determine the contribution of cells residing within the tissue, irrespective of those that may infiltrate in response to allergen exposure *in vivo*.

4.2 Discussion of results

Germline transcription through the Iε region of genomic DNA precedes the induction of productive or mature ε RNA (Cε+/Iε-) and IgE synthesis [Gauchat et al, 1990]. As such, using riboprobes to detect Iε RNA allowed for the identification of B cells undergoing this event. When nasal mucosal tissue from patients with seasonal allergic rhinitis was examined out of season, there were a fair number of cells expressing Cε RNA, but only a few Iε+ cells. This would imply that some B cells had undergone isotype switching to IgE before our investigation, from prior allergen exposure. That there were some Iε RNA+ cells may be attributed to the fact that a portion of the patients were allergic to house dust and as such some cells were undergoing ε germline

transcription at the time of biopsy. Nonetheless, there were increased numbers of cells expressing both Iε and Cε RNA in tissue obtained from placebo-treated patients following *in vivo* allergen exposure, *i.e.* either by allergen challenge or natural exposure. Since the ε germline trancript also encodes the Cε gene sequence, the increase in the number of Cε RNA+ cells may be due to resident B lymphocytes already switched to IgE production, to infiltrating IgE-producing cells or to B cells undergoing ε germline transcription. Germline ε transcripts are not present within cells that have previously undergone switching to IgE [Gauchat et al, 1990], therefore, the fact that there were more cells expressing Cε than Iε RNA following allergen challenge would indicate synthesis of productive ε RNA (Cε+/Iε-). Indeed, double hybridization experiments did demonstrate that 35% of the Cε+ were Iε-.

Using antibodies against CD20, a significantly higher number of B lymphocytes was detected within nasal mucosa obtained from seasonal allergic rhinitics out of season as non-allergic controls. However, there was no increase in the number of these cells following allergen exposure, either *in vivo* or *ex vivo*. These findings suggest that there is a constitutive B lymphocyte population and, in conjunction with the rise in Cε and Iε RNA expression, indicate that they are under local regulation. It could be argued that following *in vivo* allergen exposure the increased number of cells expressing these transcripts was due to infiltrating B cells, those that may go undetected by the CD20 assay. However, the fact that there were also increases within tissue exposed to allergen *ex vivo* confirms that the local B cell population is responsible, at least partially, for the observed elevations in Cε and Iε RNA expression. While IgE protein and mRNA have been detected in nasal secretions and lavage fluid [Huggins et al.

1975; Merrett et al, 1976; Illum et al, 1978; Diaz-Sanchez et al, 1994a; 1997; Fujieda et al, 1998], the present work is the first to clearly demonstrate synthesis of RNA coding for the ε gene within nasal mucosal tissue.

CD20 is expressed by resting and memory B lymphocytes [Zola et al, 1987; Pascual et al, 1994] but not plasma cells [Harada et al, 1993; Arpin et al, 1995; 1997], indicating that the B cells detected here are of memory and/or resting phenotype. It would appear that the majority of cells synthesizing ε RNA transcripts following allergen exposure were memory cells, since 95% of the Cε+ cells were co-localized to CD20. Although there were also Cε+ cells within sections of tissue obtained outside the season, virtually no cells co-expressing IgE protein and CD20 immunoreactivity were observed. This may be a consequence of low levels of IgE expression, secretion rather than intracellular accumulation of a major portion of the product or alternatively there may be very few IgE+ memory B cells at this site between seasons. From the data shown here, there also appeared to be a lack of IgE+ plasma cells (CD38+) within nasal tissue obtained outside the season. This is not surprising, since terminally differentiated plasma cells are considered short lived and as such not to dwell within the tissue from season to season. Furthermore, unlike the CD20+ cells, plasma cells are no longer responsive to allergenic stimulation and are thought to have shifted to synthesizing primarily secretory protein, which is less likely to co-localize to CD38+ cells. The inability to detect IgE+/CD38+ cells is consistent with previous work by Bonnefoy and colleagues, who reported only occaisional IgE+ plasma cells by

ELISPOT but an ample amount of specific-IgE in supernatants from murine lung cells cultured with antigen [Chvatchko et al, 1996].

B cells committed to IgE synthesis do not express ε germline transcripts [Gauchat et al, 1990]. As such, the increased number of cells expressing Iε RNA following allergen exposure is most likely derived from a pre-switched B cell population. IgM+ B cells and IgM itself have been detected within nasal secretions and tissue of patients with perennial allergic rhinitis [Illum et al, 1978]. Furthermore, a subset of naive IgM+IgD+CD27- B cells have been reported to comprise ~60% of peripheral blood B lymphocytes [Klein et al, 1998]. Moreover, since only 37% of CD20+ cells in the nasal mucosa were expressing Cε RNA, there is some 63% that may be committed to the synthesis of other immunoglobulins or pre-switched B cells. Further studies are required to better characterize the population(s) of B lymphocytes within allergic nasal mucosa.

In both *in vivo* and *ex vivo* allergen challenge experiments, increases in Iε RNA were seen within 24 hours. Rapid synthesis of ε germline transcripts was initially observed by Gauchat *et al.*, who demonstrated their presence as early as 2 hours following *in vitro* stimulation [Gauchat et al, 1990]. More recently, Toellner *et al.* [Toellner et al, 1996] have shown that γ_1 germline transcripts increase by almost 7 fold in splenic B cells of sensitized mice within 12 hours of *in vivo* challenge. As such, the observed increase in Iε RNA within 24 hours was not an unexpected finding. The current work also demonstrates the presence of cells synthesizing productive ε RNA (Cε+/Iε-) within this time period. Whether these transcripts are the direct result of *de*

novo class switching or to cells already committed to IgE synthesis has not been investigated, however, previous studies using peripheral blood mononuclear cells cultured with IL-4 and CD4+ T cells have shown that productive ε mRNA does not appear until day 7 of culture [Gauchat et al, 1990]. As such, it would seem that B cells previously committed to IgE production are the source of productive ε mRNA in the current experiments. On the other hand, the kinetics of switch recombination have not yet been determined *in vivo*. Using the explant technique, factors that are potentially important to cell function, like co-stimulatory molecule and cell-cell interactions remain intact and as such conditions are much closer to physiologic. It is possible that within this environment the kinetics of productive ε mRNA synthesis may be faster than what has been previously observed in cell culture experiments [Gauchat et al, 1990]. However, there was no negative correlation between Iε+ and Cε+Iε- cells following culture with allergen, indicating that Cε+Iε- cells most likely underwent switching prior to the study. PCR primers that span a conserved sequence of all Jh genes and the Cε gene segment have been used to identify mature ε mRNA (VDJ-Cε) [Riches et al, 1998] and B cells that have undergone isotype switching to IgE have been detected by amplifying the expelled DNA switch circles (Sμ-Sε) [Zhang et al, 1994]. Further study is required, however, to determine the expression kinetics of these products generated as a result of switching, *in vivo*.

While this thesis contends that there are B cells within the nasal mucosa which commit to IgE production locally, it does not exclude the possibility that some cells switch elsewhere and migrate to the tissue as such. Indeed, it has been demonstrated

here that although the counts are significantly lower than within nasal mucosa, there are Iε and Cε RNA+ cells in cytospin preparations from peripheral blood obtained from patients following seasonal exposure. This could be due to infiltration of B cells in the process of switching to IgE. Alternatively, it is also possible that some of the B cells within the nasal tissue may move into the blood following local switching. In any case, the fact remains that the number of cells expressing Iε and Cε RNA did increase following *ex vivo* exposure to allergen, verifying that at least some B cells undergo ε germline transcription locally.

To address the issue of whether the response to allergen was specific, the expression of Cγ RNA was also examined. The concentration ratio between IgG and IgE in serum of non-atopic individuals is more than 100,000:1 [Turner et al, 1996]. The current work demonstrates that the number of cells expressing Cε RNA and Cγ RNA within tissue obtained prior to allergen exposure was comparable, further emphasizing that the isotype ratio (IgG:IgE) is distorted in this patient group. Within nasal mucosal tissue obtained from individuals that received *in vivo* allergen challenge there was a significant reduction in the number of Cγ RNA+ cells, while there was no change within tissue from patients exposed to allergen during the season or by culturing it with allergen-treated medium. The fact that the number of Cγ+ cells did not simply increase following allergen exposure, as the number of cells expressing Cε and Iε RNA did, substantiates the specificity of this detection system. However, it is notable that the trend was not similar following all three exposure protocols. Possible explanations for these results include the fact that *in vivo* challenge, an acute exposure to allergen, may have

induced a more massive allergic response than the gradual exposure patients experience during the pollen season. In mice, it has been reported that at least 10 times more IL-4 was required to induce ε than γ_1 germline transcription [Snapper et al, 1988]. Indeed, it is shown here that *in vivo* allergen challenge was associated with an increase in IL-4 expression, significantly more than following natural exposure. As such, some B cells may have been induced to switch to the IgG_4 isotype instead of IgE during natural exposure, accounting for the level of total RNA coding for IgG ($C\gamma$ RNA) not being significantly reduced. The number of cells expressing $C\gamma$ RNA was also unchanged following *ex vivo* allergen challenge. This may be due to the fact that there was no contribution from infiltrating cells in this system, possibly leading to a lower concentration of IL-4 which may allow some B cells to switch in favor of IgG_4 instead of IgE. Furthermore, the fact that only 37% of CD20+ cells were $C\varepsilon$+, would imply that there may be B cells within this tissue committed to the production of other secondary immunoglobulins, including IgG_4.

Baseline levels of IL-4 and IL-13 mRNA+ cells were present within allergic tissue obtained out of season, which is consistent with previous studies [Masuyama et al, 1994; Ghaffar et al, 1997]. Furthermore, allergic nasal tissue cultured in medium alone exhibited 2 fold more cells expressing IL-4 mRNA than similarly treated non-allergic tissue. This difference in baseline levels was not observed for the number of cells expressing IL-13 mRNA, which suggests that the presence of IL-4 may be a predisposing factor for allergy. Multiple allelic forms of the human IL-4 promoter are known to exist within the general population and recently a nucleotide transition from

A to G, associated with increased transcription factor binding affinity and over-expression of the IL-4 gene, has been demonstrated. It is not known, however, whether allergic individuals exhibit an increased frequency of these allelic forms [Song et al, 1996].

The number of cells expressing IL-4 and IL-13 mRNA was increased within allergic tissue after all three exposure protocols. While this has previously been observed following *in vivo* allergen challenge [Ying et al, 1994; Ghaffar et al, 1997], an increase in IL-4 mRNA as a result of natural exposure is novel. Elevated levels of both IL-4 and IL-13 are recognized as characteristic of allergic respiratory mucosa, however, this has primarily been considered a consequence of inflammatory cell infiltration. Here it is shown that there are significantly more cells expressing IL-4 and IL-13 mRNA following *ex vivo* stimulation, providing direct evidence for local cytokine synthesis. While this has been demonstrated previously using PCR analysis [Okamoto et al, 1993], the current work has employed *in situ* hybridization for the detection of cytokine mRNA. Unlike PCR, this technique is performed on intact tissue sections and as such it is possible to ascertain morphological information and to phenotype the cells expressing the RNA product in question. Using a combination of *in situ* hybridization and immunocytochemistry, it has been demonstrated here that a significant proportion of the IL-4 and IL-13 mRNA+ cells within allergic tissue cultured in allergen-treated medium were co-localized to T cells and mast cells. These two cell types appeared to account for all the IL-4 mRNA+ cells within the tissue, consistent with what has been observed previously following *in vivo* allergen challenge [Ying et al, 1994]. However,

local T cells were the source of only 44% of the total number of IL-13 mRNA+ cells. This is not in agreement with *in vivo* allergen challenge data indicating these cells were responsible for 77% of IL-13 mRNA [Ghaffar et al, 1997], which points to the fact that a fair amount of IL-13 within allergic nasal tissue is attributed to infiltrating, rather than local, T cells. Furthermore, only 62.5% of the total number of cells expressing IL-13 mRNA within *ex vivo* challenged nasal tissue was associated with T cells and mast cells, underlining the importance of other cellular sources of this cytokine. Indeed, it was observed here that a large proportion of the IL-13 mRNA+ cells that were not co-localized to T cells or mast cells were of a morphology consistent with the macrophage. This is in accord with a recent report demonstrating the production of this cytokine by alveolar macrophages [Hancock et al, 1998]. In light of the observed increase in Iε RNA following *ex vivo* allergen challenge, it appears that IL-4 and IL-13 made locally was capable of inducing ε germline transcription in neighboring B cells. These findings demonstrate that the local synthesis of IL-4 and IL-13 may regulate the generation of IgE within nasal mucosal tissue itself.

Within 24 hours of allergen challenge, either *in vivo* or *ex vivo*, there was no correlation in the number of cells expressing IL-4 and Iε RNA. Since IL-4 is considered to induce ε germline transcription [Stavnezer et al, 1988; Rothman et al, 1990; Gauchat et al, 1990; Punnonen et al, 1995; Ezernieks et al, 1996] a correlation between the expression of the two transcripts was anticipated. While the point counting method used in this thesis determines the number of cells expressing the

mRNA of interest, it does not give any information on copy number. As such, whether individual cells were making more cytokine after allergen exposure than before could not be determined. The apparent lack of a relationship between IL-4 mRNA and Iε RNA expression may be due to this phenomenon. Alternatively, it could be attributed to the fact that the expression kinetics of these transcripts are different, since it is the IL-4 *protein* that induces Iε RNA expression, not the mRNA. However, within tissue from patients naturally exposed to allergen a significant correlation between the number of cells expressing IL-4 mRNA and Iε RNA was observed. This may be a function of long-term exposure to allergen and indicates the importance of IL-4 production from both resident and infiltrating cells.

Patients that received pre-treatment with fluticaone propionate (FP) exhibited an almost complete inhibition in the increase in Cε+and Iε+ cells. Since there were virtually no Iε+ cells prior to allergen exposure, it appeared that FP treatment arrested ε germline transcription. While the increase in Cε+ cells was also inhibited, there was still a similar number of these cells as within tissue obtained prior to allergen exposure. This would suggest that a reduced number of Cε+ cells was most likely a result of interupting ε germline transcription. Other anti-inflammatory agents such as nedocromil sodium and sodium cromoglycate have been shown to inhibit the production of Sμ-Sε switch circles, however, they appear to have no affect on the expression of IL-4-induced ε germline transcripts [Loh et al, 1994; 1996]. As such, the present findings are the first to demonstrate the inhibition of ε germline transcription by anti-inflammatory reagents. Steroid treatment was also associated with a reduction in the number of IL-4 mRNA+

cells. While it has been previously demonstrated that pre-treatment with intra-nasal steroid attenuates the increase in the number of cells expressing IL-4 mRNA following allergen challenge [Masuyama et al, 1994], the present work is the first to verify these results in patients naturally exposure to allergen during the pollen season.

There are a number of mechanisms by which steroids may affect the allergic process within the nose. Since FP has been shown to have no systemic bioavailability at the dose utilized (200 μg/day) [Harding et al, 1990], it most likely acts directly on the resident inflammatory cells. Acute exposure to allergen, such as *in vivo* challenge, results in both early and late nasal symptoms considered to be mediated primarily by mast cell- and T cell-derived factors, respectively [Varney et al, 1992; Durham et al, 1992; Rak et al, 1994]. The early, mast cell-derived, presence of IL-4 is thought to induce the Th2 phenotype [Wang et al, 1999]. It has been observed here and by others [Ying et al, 1994] that mast cells account for a considerable proportion of the IL-4 mRNA+ cells within allergic nasal tissue. Since steroids inhibit stem cell factor expression [Kim et al, 1997], which is required for retaining mast cells from apoptosis [Iemura et al, 1994], it seems likely that steroid-induced mast cell death may be one of the mechanisms responsible for the observed reduction in IL-4 mRNA+ cells. Steroids also inhibit T cell production of IL-4 [Byron et al, 1992; Umland et al, 1997]. Here it is shown that T cells are the major source IL-4 mRNA, indicating that arrest of IL-4 synthesis may also be an important pathway. As such, it would appear that the inhibited expression of Iε and Cε RNA is due to a diminished presence of IL-4, however, to be conservative one can only report the concomittant reduction in the number of cells expressing these factors. Still another possible mechanism by which steroids inhibit

ε germline transcription is by interupting DNA binding of NFκB, which is induced by both IL-4 and CD40 signalling [Lalmanach-Girard et al, 1993; Berberich et al, 1994; Delphin et al, 1995; Iciek et al, 1997], to its response element within the ε promoter. Steriods induce the expression of IkB, which causes NFkB to dissociate from this site and move out of the nucleus [Scheinman et al, 1995; Barnes et al, 1997].

Close examination of the data from individual patients demonstrates that there was a somewhat variable response to steroid treatment. Although nasal tissue from the majority of patients was seen to have reduced numbers of cells expressing Cε, Iε and IL-4 RNA when pre- and post-exposure biopsies were compared, some patients exhibited persistent increases. There are a number of possible reasons for this variability. One of the most obvious is a lack of compliance. Despite the efficacy of this steroid regime, as a pre-treatment rather than for rescue purposes, it is nonetheless counter-intuitive for many patients and may in some cases be responsible for a 'less than regular' steroid-intake. These may be the patients that demonstrated increases in the number of cells expressing Cε, Iε and IL-4 RNA. Another more complex possibility is the fact that there may be heterogeneity within this population of allergic rhinitics. Some patients may be more responsive to steroid treatment than others. This is a phenomenon previously observed in asthma, as some patients respond incredibly well to steroids while others appear to be insensitive [Carmichael et al, 1981], suggested to be due to a dysregulation in Th2 cytokine synthesis [Leung et al, 1995]. It is also possible that the pathogenesis of allergic rhinitis is not the same in all patients and as such they may not all be similarly susceptible to steroid

treatment. In any case, the mean number of cells expressing these RNA transcripts was not increased in patients pre-treated steroids.

In vitro, the synthesis of IgE is potentiated by the presence of hydrocortisone [Wu et al, 1991] and surface IgE positive B cells (sIgE+) produce IgE in response to this hormone in an IL-4-independent manner [Kimata et al, 1995]. These studies indicate that steroids alone may activate previously switched B cells to produce IgE. Indeed, from these and other similar observations [Hiratsuka et al, 1996], it has been proposed that steroid-associated symptom improvement may be attributed, not only to the downregulation of cytokine synthesis and subsequently of *de novo* switching to IgE, but also to increased production of polyclonal IgE that could compete with antigen-specific IgE for Fcε-binding sites. However, the present work demonstrates that the number of cells expressing Cε and IL-4 mRNA were not increased within patients treated with FP prior to allergen exposure, indicating the unlikelyhood that polyclonal activation of B cells was a factor in the observed clinical improvement. Furthermore, these observations reinforce the supposition that local B cells are induced to undergo ε germline transcription via an antigen-dependent mechanism.

Fluticasone propionate appeared to be ineffective on cells already committed to IgE synthesis, highlighting the ultimate importance of priming and the possibility that this event may be irreversible. Maternal atopic status is currently considered a possible risk factor for the development of allergy [Jones et al, 1998]. While the presence of Th2 cytokines at the materno-fetal interface is regarded as important for successful pregnancy [Wegmann et al, 1993], the deficiency in Th1 cytokines such as IFN-γ

within cord blood mononuclear cells has been suggested to be predictive of atopic disease [Warner et al, 1994; Kondo et al, 1998]. From this work, the fine balance between Th1 and Th2 cytokine expression within the intra-uterine environment has become apparent. As such, altering this balance may influence the development of allergy [Jones et al, 1998]. Once initiated, a predominantly Th2 environment may confer a highly powerful, difficult to reverse, positive feedback mechanism which could lead, if left untreated, to a life-long imbalance in the Th2/Th1 cytokine ratio. This would be consistent with the current understanding of immunotherapy, a conscious effort to shift the cytokine profile toward Th1 expression, which is reported to require 3 - 5 years for optimal effectiveness [Durham et al, 1999].

4.3 Implications of these results

The present results indicate that there is B cell committment to IgE within allergic nasal mucosal tissue in response to allergen exposure. As such, it is possible that this IgE may bind to FcεRs on antigen presenting cells (APC) and lead to local antigen presentation to T cells. Although this is an event considered to occur within the secondary lymphoid organs, IgE+/MHC II+ Langerhan's cells have been identified within the nasal mucosa of patients with allergic rhinitis [Godthelp et al, 1996]. Furthermore, these cells have been observed to induce stable IL-4-producing T cell lines in response to antigenic stimulation [Hauser et al, 1989]. Antigen presentation by B cells has also been seen to induce T cell production of IL-4 [Secrist et al, 1995], possibly due to their own production of this cytokine [Bastien et al, 1999]. Furthermore, both antigen-specific and non-specific B cells may detect antigen, via

sIgE (the BCR) and IgE-CD23 complexes [van der Heijden et al, 1993], making these cells extremely sensitive to low concentrations of antigen. It is well recognized that B cells present antigen to T cells within lymphoid tissues [Liu et al, 1996]. In view of the fact that there is a population of B lymphocytes within the nasal mucosa, it is possible that these cells may also present antigen to T cells at this site.

In either case, local antigen presentation would induce resident T cells to express IL-4 and CD40L simultaneously, providing the necessary signals for B cells to switch to IgE. It would seem that presentation by the B cell may prove more efficient than the three-way APC-T cell-B cell interaction. However, T lymphocytes express CD40L only transiently after activation and other inflammatory cells, such as the mast cell, are characterized by constitutive expression of this surface molecule [Gauchat et al, 1993]. Moreover, Ganzer *et al.* have reported the interaction between mast cells and B cells within lymphatic tissue [Ganzer et al, 1988]. Taken together with the co-localization of IL-4 and IL-13 mRNA to these granulocytes within the nasal mucosa, shown here as well as elsewhere [Ying et al, 1994; Ghaffar et al, 1997], it appears that mast cells may also participate in driving B cell isotype switching to IgE.

Germline transcription has been shown to require DNA synthesis [Lundgren et al, 1995]. Although the number of divisions for switching to IgE is not known, it is estimated that at least 3 cell cycles are required for murine B cells to express IgG_1 [Hodgkin et al, 1996]. This indicates that, in contrast to the present findings, ε germline transcription is accompanied by B cell proliferation and therefore an increase in B cell number. Despite the general consensus that CD20 is a reliable pan B cell

marker, there are repors that this surface molecule may be lost after B cell activation [Zola et al, 1987]. The apparent lack of increase in the number of B cells within the nasal tissue following allergen exposure may be due to this phenomenon. Since CD19 is retained on activated B cells and is expressed earlier than CD20 [Zola et al, 1987], it is possible that detection of this surface antigen may be a better marker of B cells. Alternatively, a substantial number of cells may be lost to migration across the epithelium, consistent with our observation of intra-epithelial B cells and previous reports of their presence within nasal lavage fluid [Fujieda et al, 1998]. Increased numbers of B cells as a result of isotype switching within the nasal tissue would create, at first glance, a problem for interpreting the data obtained from patients who underwent *in vivo* allergen exposure. However, the fact that there were also higher numbers of cells expressing ε RNA following allergen exposure in the closed explant system, which did not allow for cellular infiltration, confirms that there was local synthesis. The prospect of B cell clonal expansion in the nasal mucosa is an interesting area of investigation.

Collectively, these results evoke the question of whether lymphoid tissue may form within human nasal mucosa, as it does within the gut. Peyer's patches represent the primary site for uptake and presentation of ingested antigens in the intestine and are known sites of germinal center formation, somatic mutation and isotype switching. While Iα RNA transcripts have been identified within Peyer's patches of the rat [Weinstein et al, 1991], the present work is the first to demonstrate the expression of Iε RNA within peripheral tissue in the human. As such, the point quickly becomes whether the B cell switches to IgE outside the germinal center or if nasal associated

lymphoid tissue (NALT) forms at this site. Although germinal center development has been shown to occur within the lungs of mice following antigen challenge [Chvatchko et al, 1996], there have been no reports on analogous structures within human nasal mucosal tissue. Since biopsy specimens allow one to observe only a small area of the entire nasal mucosa and individual germinal centers are considered to be transient, existing for only a few weeks [MacLennan et al, 1994], it is possible that these structures have been overlooked in the past. On the other hand, it seems less likely that within nasal tissue obtained from patients out of season, increased numbers of cells expressing Iε RNA only 24 hours after allergen exposure would be B cells derived from newly developed germinal centers. Indeed, histological observations of this work indicate that the cells expressing Iε RNA stand alone or in clusters of three or four cells, not in B cell-rich areas, as is characteristic of a germinal center. However, that is not to say that the cells expressing Iε RNA are not of a germinal center phenotype. These cells can be identified by the expression of the surface antigen CD10 [Lebecque et al, 1997]. As such, it would be interesting to examine B cell expression of this marker within the nasal mucosa of patients with full blown symptoms on a regular basis. To date, however, it remains to be seen whether germinal centers form at this site.

The implications of this thesis work, as highlighted above, focus attention on the fact that there are many unanswered questions regarding the capability and role of the nasal mucosa in the pathogenesis and propagation of the allergic response. With this nasal mucosal explant system, however, many of these implications can be

investigated. While cell culture has greatly advanced our understanding of cell and cytokine function, is it limited in the respect that it lacks important elements of the *in vivo* situation, like cell-cell and cell-matrix interactions as well as complex inter-cytokine networking. The strength of the explant technique is two-fold. Firstly, it side-steps the difficulty of re-creating an '*in vivo*-like' environment and secondly it allows for the delineation of local *vs.* systemic events. Furthermore, this technique holds potential for evaluation of pharmacologic agents, as it provides an intermediate system between *in vivo* animal studies and human clinical trials.

4.4 Therapeutic implications.

The findings of this thesis emphasize the importance of local, in addition to systemic, measurements in determining a patient's allergic status. In extrapolation of these results and previous work demonstrating the expression of ε RNA within ethmoidal mucosa of non-allergic patients with chronic sinusitis [Ghaffar et al, 1998], it appears that some patients may experience a more prominent local response which could lead to a *mis-diagnosis* of 'non-allergic'. As such, this work calls into question the current procedures for determining atopic status, *i.e.* skin reactions to allergen and specific IgE within the serum, and indicates the need to supplement these tests by intra-nasal challenge in patients that manifest the symptoms characteristic of allergic rhinitis but are not positive with standard testing. Similarly, local switching and production of IgE may also help explain site-restricted manifestations of allergy, such as allergic rhinitis and asthma.

Recent advances in the treatment of allergic disease include leukotriene receptor antagonists as well as a humanized monoclonal anti-IgE Ab (E25). Clinical trials have

demonstrated that both these reagents are highly effective in the suppression of allergen-induced early and late phase symptoms of allergic asthma [Taylor et al, 1991; Fahy et al, 1997; Boulet et al, 1997], but have failed to provide significant relief of nasal symptoms in patients with seasonal allergic rhinitis [Casale et al, 1997; Pullerits et al, 1999]. In view of this thesis work, since leukotriene receptor antagonists are an oral medication and the clinical trials with E25 were carried out using intravenous injection [Casale et al, 1997], it is conceivable that their failure to improve nasal symptoms may be due to route-of-administration. Local application of these reagents, such as a nasal spray, may prove more effective and is certainly worth investigation.

While steroids are well recognized in the treatment of allergic rhinitis, their effects are by no means cytokine specific. Since it has been determined that IL-4 and IL-13 are essential for initiating isotype switching to IgE, inhibiting ε germline transcription by interfering specifically with the action of these cytokines is a more elegant approach. Mutant IL-4 molecules that form unproductive complexes with IL-4Rα have been shown to antagonize IL-4 and IL-13 function, abrogate the humoral immune response to allergen as well as the development of allergic symptoms *in vivo* in mice [Grunewald et al, 1997; 1998]. Furthermore, although both these cytokines induce germline transcription from the Iε region [Del Prete et al, 1988; Rousset et al, 1991; Gauchat et al, 1990; Punnonen et al, 1995], the CD40 molecule must also be activated [Noelle et al, 1992]. Blocking this interaction with soluble CD40 may provide an effective method for local prevention or downregulation of IgE production within the nasal mucosa [Nonoyama et al, 1993]. In addition to the production of IgE, IL-4 and IL-13, there is also

a strong presence of eosinophils and eosinophil-associated mediators such as IL-5 and eotaxin within the nasal mucosa during an allergic response to allergen [Ying et al, 1993; Minshall et al, 1997b]. For this reason, it would appear that although agents which inhibit IL-4/IL-13 interaction with the ε promoter may arrest B cell switching to IgE, it is likely that the most effective treatments of the future will be a combination of inhibitory agents that target the multiple pathways involved in the pathogenesis of the allergic inflammatory response.

4.5 Future studies

There are a number of ideas for further research which arise from the findings of this thesis:

1) From a clinical perspective, it would be interesting to examine the local expression of Cε and Iε RNA in patients that have nasal symptoms synonomous with 'allergic' rhinitis but appear to be negative on skin testing and RAST measurement. From the current findings it could be hypothesized that these patients may experience a more prominent local response and may therefore exhibit a higher number of cells expressing ε RNA than tissue from allergic patients with similar symptomatology as well as elevated serum IgE levels.

2) It has been shown here that culturing nasal mucosal tissue for a period of 24 hours results in no apparent loss of tissue viability, indicating the feasibility of more extended culture periods. Long-term culture would provide the proper time frame in which to examine increases in *de novo* IgE protein, which reportedly requires 7 days in liquid culture [Gauchat et al, 1990]. PCR primers to identify Sμ-Sε switch circles and/or

VDJ-Cε mRNA [Zhang et al, 1994; Riches et al, 1998] would help to further delineate the kinetics of class switch recombination to IgE. In addition, long-term culture would also provide a system in which to observe whether germinal centers and NALT actually form within the nasal mucosa in response to prolonged allergen exposure.

3) Further characterization of this B cell population is also important. The expression of CD23 and CD27 have been reported to marker pre-switched and somatically mutated B cells, respectively [Bonnefoy et al, 1996; Klein et al, 1998]. As such, it would be interesting to co-localize the expression of these markers with CD19, before and after allergen exposure. Furthermore, the question of whether there is clonal expansion of B lymphocytes within the nasal mucosa in response to allergen has also arisen. Co-localization experiments using CD19 and a marker of proliferation such as proliferating cell nuclear antigen (PCNA) would address this point.

4) The present work also sheds some light on potential avenues for therapeutic intervention. IL-12 is increased following steriod treatment [Naseer et al, 1997], immunotherapy [Hamid et al, 1997a] and has been shown to induce IgG_{2a} while inhibiting IgE expression in stimulated murine splenic B cells [Yoshimoto et al, 1997; Matsuse et al, 1999]. On the basis of these studies, it would be interesting to culture nasal mucosal explant tissue with rhIL-12 prior to addition of specific allergen. This may induce B cells to commit to IgG_{2a} and thereby circumvent the production of IgE in response to allergen. On a similar note, since IL-10 has been suggested to favor IgG_4 over IgE synthesis [Jeannin et al, 1998a] it would also be interesting to culture nasal mucosal tissue with rhIL-10 and specific allergen to investigate whether there is a significant shift in the IgG_4:IgE ratio.

4.6 Summary and conclusion

In summary, this thesis provides evidence of ε germline transcription within nasal mucosal tissue and indicates that this event may be regulated by *in situ* cytokine synthesis. Using three methods of exposure, it was demonstrated that the nasal mucosa of individuals with seasonal allergic rhinitis is characterized by an increase in the number of cells expressing ε RNA as well as IL-4 and IL-13 mRNA, cytokines important for ε germline transcription and production of IgE. In addition, it was also shown that these increases were abrogated by pre-treatment with topical corticosteroids. Increased expression of IL-4, Cε and Iε RNA in response to allergen exposure, in the absence of a significant difference in B cell number, was highly suggestive of local ε germline transcription within the nasal mucosa. Furthermore, *ex vivo* exposure to allergen also resulted in an increase in the number of cells expressing Cε and Iε RNA, which provided clear evidence that this was indeed an local event. This work demonstrates that the nasal mucosa is a viable locale for B cell commitement to IgE synthesis and the production of IgE-inducing cytokines. As such, these findings indicate that the nasal mucosa may be a primary site for regulation of the allergic response in patients with seasonal allergic rhinitis.

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