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Studies Investigating Peripheral Blood Derived Cells That Express The High Affinity Receptor For Immunoglobulin E (Fcε RI) In Allergic Disorders

by

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ABSTRACT

SCHOOL OF MEDICINE

Doctor of Medicine

STUDIES INVESTIGATING PERIPHERAL BLOOD DERIVED CELLS THAT EXPRESS THE HIGH AFFINITY RECEPTOR FOR IMMUNOGLOBULIN E (Fcε RI) IN ALLERGIC DISORDERS

By Bhupinder Singh Sihra

It is just forty years since the identification of immunoglobulin E (IgE) as the reagin responsible for allergen induced immediate hypersensitivity reactions. IgE exerts its biological actions through the binding of its Fc fragment to specific Fc receptors on effector cells. There are two predominant Fc receptors for IgE – FcεRI, which has a very high affinity for IgE and FcεRII, which shows less avid binding. For much of the first two decades after the identification of IgE, it was thought that FcεRI expression was limited to mast cells and basophils and that IgE binding to other cell types such as B-lymphocytes and antigen presenting cells (APCs) was mainly due to FcεRII . However with major advances in characterisation and functional knowledge of FcεRI, particularly in the last fifteen years, it has become apparent that FcεRI can be expressed on several more cell types that may be involved in initiation and maintenance of allergic inflammation – including APCs (monocytes and dendritic cells) and possibly eosinophils.

The research described in the four papers forming this thesis was completed during this period and evaluated FcεRI expression on different cell types, their potential roles in allergen induced inflammatory responses and whether successful therapeutic strategies for allergic disorders may involve actions on FcεRI+ cells. The relative expression of FcεRI on peripheral blood basophils, monocytes and eosinophils from atopic and non-atopic subjects and any relationship with serum IgE concentrations was assessed in the first paper. The second study examined a potentially important role for basophils as a cellular source of rapidly releasable IL-4 which may help initiate allergen induced T_H2 responses. The next study investigated the possible effects on allergen induced early and late asthmatic responses of the immunosuppressive drug cyclosporin A which had been shown both to inhibit mast cell and basophil degranulation and cytokine secretion (particularly by CD4+ T-cells). The final study evaluated FcεRI expression on these cell types as well humoral factors (e.g. seasonal changes in allergen specific IgG and IgE) in subjects who, after 3 to 4 years of grass pollen immunotherapy, had continued on either active or placebo immunotherapy for a further 3 years.

A historical perspective explaining some of the reasons the studies were done is provided in the introductory chapter whilst the discussion chapter at the end reviews how many of the findings of the study have evolved in subsequent years right up to the present day and finishes off with a brief synopsis of how rapidly increasing knowledge of the regulatory functions of dendritic cells (expressing FcεRI and often monocyte derived) has resulted in better understanding of the mechanisms of allergen specific immunotherapy and is leading to more effective treatment modalities.

CONTENTS

AUTHOR'S DECLARATION

This thesis is submitted for the degree of Doctor of Medicine under Regulation 21(b) of the "Regulations for the Degree of Doctor of Medicine" of the University of Southampton. This regulation covers the requirements for the award of the DM degree by submission of published works and states:

"21b. Any graduates who hold a Consultant or equivalent position, or who work in General Practice, may apply for the award of the degree by submission of published works. Such works should be broadly comparable to a DM thesis, as specified in regulation 24 below. The normal requirement would be a minimum of four peer-reviewed papers in respected journals which form a coherent body of work".

My proposal, including the papers to be considered for the degree award, was accepted by the School of Medicine in late 2006. The proposal included three peer reviewed published papers and also one unpublished paper (that presented unpublished data from a clinical study which has been published) which, after due consideration, was accepted to be relevant to the thesis.

I also presented the following statement which was accepted by the School of Medicine:

Statement Confirming The Extent Of My Participation In The Work Described In The Papers Submitted

I can confirm that I was fully involved in all the projects described in the papers to be included in this submission. I was at least an equal contributor, with my co-author Dr Onn Min Kon, to the work done in all the studies involved.

A more specific breakdown of my role in the 4 main studies:

- *Study 1: I performed the preliminary work developing the flow cytometric method used in this study and then, together with Dr Kon, planned and performed the study. I was involved at all stages – from recruiting and characterising suitable subjects, obtaining blood samples, processing blood samples, gathering and analysing data and writing and submitting the paper.*
- *Study 2: Once again I was a full and equal investigator involved in ever step, from planning to completing the study and successfully submitting the paper.*
- *Study 3: This was my initial research project and I was the main investigator from the outset. Although the draft protocol had been drawn up before I joined the Department, I drew up the Ethics Committee submission and successfully completed the study. Dr Kon assisted me in the latter stages of this study.*

- *Study 4: This study was a collaboration from the outset as it involved processing samples obtained from subjects participating in a long term study in the Department. However I was involved in drawing up the study protocol and the laboratory work in all the subjects. Similarly, I was an equal partner in the analysis of the data.*

I can also confirm that for all these papers Dr Kon and I set down and wrote them together. Prior to writing these papers we prospectively (and randomly) decided who would be the first name on the published paper and agreed that in the (almost unheard of) event of a disagreement on emphasis which could not be resolved, the nominal first name would get final editorial authority.

The statements I have made can be corroborated by Dr Kon (who is one of my referees) and, if necessary, by Professor A B Kay who was the head of the Department of Allergy and Clinical Immunology at the time (and, as such, ultimately the supervisor of all these studies).

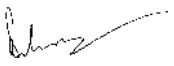
Dr Bhupinder Singh Sihra
06 September 2006

[References:

1. *Expression of high affinity IgE receptors (FcεRI) on peripheral blood basophils, monocytes and eosinophils in atopic and non-atopic subjects: Relationship to serum immunoglobulin E concentrations. BS Sihra, OM Kon, JA Grant, AB Kay. J Allergy Clin Immunol 1997; 99: 699-706*
2. *Unstimulated peripheral blood basophils in atopic and non-atopic subjects express intracellular interleukin-4: detection by flow cytometry. OM Kon, BS Sihra, SJ Till, JA Grant, AB Kay. Allergy 1998; 53: 891-896*
3. *The effect of cyclosporin A on the allergen-induced late asthmatic reaction. BS Sihra, OM Kon, SR Durham, SM Walker, NC Barnes, AB Kay. Thorax 1997; 52: 447-452*
4. *Effects of maintenance or cessation of prolonged allergen specific subcutaneous injection immunotherapy on humoral immune responses and cell surface IgE receptor expression in peripheral blood. BS Sihra, OM Kon, T Bjerke, LK Poulsen, SM Walker, SJ Till, SR Durham (unpublished paper) – draft enclosed for scrutiny (will need updating and reformulating if thesis proposal accepted)]*

I can also confirm that:

- I have not previously submitted any part of this thesis for a degree or any other qualification at this University or any other institution;
- I have acknowledged all the main sources of help;
- Where I have consulted the published the work of others, this is always clearly attributed.

Signed: 

Date: 25 May 2008

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I would particularly like to thank Professor (Emeritus) Barry Kay who was the head of the Department of Allergy and Clinical Immunology at the National Heart and Lung Institute where I completed my research. As was the case with every research fellow who has had the privilege to work for Professor Kay, my research, as well as my aptitude for research, were immeasurably improved by dynamic leadership.

I would also like to acknowledge the help and support I received from Professor Neil Barnes and Professor Steve Durham who both supervised studies that I participated in, and without whose help and direction I would not have succeeded. A special mention should also be made for Professor Chris Corrigan who was always at hand to patiently guide and instruct me (and every other research fellow in the department) in the complexities of immunology and the thrills of well planned research and willingly provided critical appraisal for papers before submission.

Finally, and most importantly, my heartfelt thanks to my friend and colleague Dr Onn Min Kon. We were a true team – often described by our colleagues as two minds working as one. Our partnership of equals allowed us to successfully overcome many obstacles, not just in the studies described in this thesis but also in the other studies we collaborated on, and the time we spent together writing the papers describing our work was truly an invigorating time – joint and equal authorship with ideas flowing, healthy discussion but remarkably few differences of opinion!

The company and help of all of the above as well as all the other members of the department at the time (too numerous to mention individually) meant that the time I spent there will always remain one of the most stimulating and memorable periods in my professional career.

Last, but certainly not least, I would like to express my gratitude to Dr Graham Roberts, Reader in Paediatric Allergy and Respiratory Medicine at Southampton University, for all the help and advice he provided whilst I was writing this thesis.

Glossary of Abbreviations

APC	antigen presenting cell
aTreg	adaptive regulatory T-cell
cDNA	complementary DNA
CD	cluster of differentiation
CsA	cyclosporin A
CTLA-4	cytototoxic T-lymphocyte antigen 4
DC	dendritic cell
EAR	early asthmatic reaction
EPR	early phase reaction
Fab	antigen binding fragment of an immunoglobulin
Fc	crystallisable fragment of an immunoglobulin
FcR	receptor for the Fc portion of an immunoglobulin
FcεRI	high affinity Fc receptor for immunoglobulin E
FcεRIα	α-chain of the high affinity Fc receptor for immunoglobulin E
FcεRIβ	β-chain of the high affinity Fc receptor for immunoglobulin E
FcεRIγ	γ-chain of the high affinity Fc receptor for immunoglobulin E
FcεRII	low affinity Fc receptor for immunoglobulin E
Ig	immunoglobulin
IgE	immunoglobulin E
IDEC	inflammatory dendritic epidermal cells
IL	interleukin
IT	immunotherapy
ITAM	immunoreceptor tyrosine-based activation motif
Ka	equilibrium association constant
mAb	monoclonal antibody
LAR	late asthmatic reaction
LPR	late phase reaction
mDC	myeloid dendritic cell
MIRR	multichain immune recognition receptor
nTreg	natural regulatory T-cell
PAG	PIPES – Albumin – Glucose buffer

PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PL	phospholipase
SCIT	subcutaneous immunotherapy (allergen specific)
SLIT	sublingual immunotherapy (allergen specific)
T_H	T helper cell subset (e.g. T_H1 or T_H2)
Treg	Regulatory T lymphocyte
TLR	Toll like receptor

Chapter 1

Introduction:
The High Affinity Fc Receptor for Immunoglobulin E in Allergic Diseases – A Brief Review

"Whereof what's past is prologue, what to come
in yours and my discharge"
The Tempest, Act II, Scene 1

1.1 Format And Context Of This Thesis

This main body of this thesis comprises of four papers all written in the late 1990s (Sihra et al. 1997a;Sihra et al. 1997b;Kon, Sihra et al. 1998; Sihra et al. 2000). The studies described in these papers all investigated either the expression of FcεRI, the high affinity Fc receptor for immunoglobulin E (IgE), on peripheral blood leucocytes or aspects of the roles of blood-derived FcεRI bearing cells play in allergic inflammation. One aim of this dissertation is to provide a historical perspective to these four studies by describing the nature and chronology of research investigating immunoglobulin E and its high affinity Fc receptor – from the discovery of IgE right up to the end of the last decade, when the research described in these four papers was undertaken. This will be the main objective of this introductory chapter. A further aim of the thesis is to provide a review of progress in the areas of research covered by each these four studies in the period since they were concluded and this will be done in the discussion in Chapter 6.

1.2 Immunoglobulin E (IgE)

1.2.1 Brief history

The story of the identification of IgE goes back to the early 1920s when Carl Prausnitz and Heinz Küstner first demonstrated the existence of a factor in the serum of an allergic patient that could passively transfer immediate skin reactivity to a non-

allergic patient (the PK test) (Prausnitz & Küstner 1921). Coca and Cooke in 1923 reported that this serum factor (or 'body') was heat labile and termed it 'reagin' (advising avoidance of the term antibody as at that stage there was "no evidence that these bodies appeared as the result of immunological stimulation") (Coca & Cooke 1923).

Despite great efforts, further progress towards identifying and characterising this reagin was frustratingly slow for the next four decades until, as has often been the case in medical science, two groups working independently made almost simultaneous breakthroughs. In 1967 Teruko and Kimishige Ishizaka (based in Denver) managed to characterise this reagin as a new class of immunoglobulin which they provisionally called the 'γE-globulin' (Ishizaka & Ishizaka 1967). At almost the same time Hans Bennich and Gunnar Johansson in Uppsala, Sweden detected an atypical myeloma protein (provisionally called IgND) which demonstrated many of the physicochemical properties of the reagin (Johansson & Bennich 1967). They further demonstrated that this IgND was present in low concentrations in serum of healthy and allergic individuals but serum concentrations were significantly higher in allergic asthmatics when compared to normal controls (Johansson 1967). In addition it was demonstrated that both whole IgND and its Fc fragment could block the PK reaction (passive transfer of skin reactivity) in human subjects (Stanworth et al. 1967;Stanworth et al. 1968).

Investigators quickly established that γE-globulin and IgND shared antigenic properties. At a World Health Organisation workshop in 1968 it was finally agreed that there was enough data available that both γE-globulin and IgND were the same protein and that this new class of immunoglobulin (termed Immunoglobulin E or IgE) was indeed the reagin first described in the 1920s (Bennich et al. 1968).

It was the discovery of immunoglobulin E that really ushered in the modern era of the science of allergy that, together with major advances in cell and molecular immunology as well as genetic techniques in the intervening four decades, has led to greatly increased understanding of allergic diseases and allergic inflammation.

1.2.2 IgE structure

Immunoglobulin E is the least abundant of the 5 immunoglobulin subclasses in serum with concentrations typically around 10^{-5} times those of immunoglobulin G, IgG. Like other immunoglobulins, an IgE molecule has 2 identical heavy (ε class) and 2 identical light (κ or λ) chains and can be cleaved proteolytically into an antigen binding fragment (Fab), composed of one constant and one variable domain of each heavy and the light chain, and a crystallisable fragment (Fc) which contains the cell receptor binding site (Bennich et al. 1968). In contrast to IgG, which has 2 constant domains in the Fc region, the Fc portion of IgE has an extra constant domain (Cε2) (*figure 1*) (McDonnell et al. 2001). As will be discussed later this extra domain has recently been shown to have important functional implications particularly related to stabilising the binding of IgE to its high affinity receptor FcεRI (McDonnell et al. 2001;Wan et al. 2002).

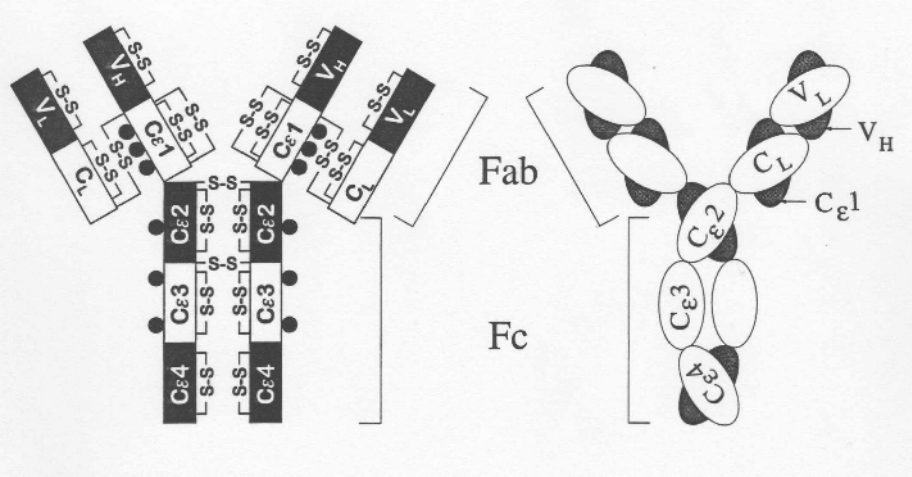

Figure 1: Representative illustrations of the polypeptide and domain structures of the IgE molecule (from B.J. Sutton and H.J. Gould, *Allergy and Allergic Diseases Ed. A. B. Kay*)

Although high resolution x-ray diffraction studies of the crystal structure of immunoglobulin E would only be available at the start of the current decade, fluorescence resonance energy transfer analyses published in the early 1990s revealed that the IgE molecule is not the simple Y-shaped structure depicted above, but is in fact a compact, tightly bent structure (Zheng et al. 1991). Further studies suggested that the Cε2 region acts as a hinge with the two Cε3 domains being twisted into a

convex loop (one Cε3 on the outside and one on the inside) (Beavil et al. 1995). Further details of the crystal structure of IgE and its interaction with the FcεRIα chain are provided in section 1.5.

1.3 Immunoglobulin E And Its Fc Receptors

Although the role of immunoglobulin E (IgE) in the initiation and maintenance of allergic inflammation is well established today, it is sometimes forgotten that it is only in the last decade and a half that there have been a number of major, and astonishingly rapid, advances in our understanding of many of the underlying mechanisms involved. The four core papers of this thesis all describe work done during this period and reflect these evolving changes in our understanding of these processes.

Previously it was known that IgE binds to the surface membrane of tissue mast cells and circulating basophils with a much higher affinity than other immunoglobulins bind to their particular receptors (FcR) on other immune effector cells (Malveaux et al. 1978;Coleman & Godfrey 1981;Pruzansky & Patterson 1986) and that IgE mediated exocytosis of mediators (such as histamine) from these two cell types was important in immediate hypersensitivity reactions (Ishizaka et al. 1973;Ishizaka & Ishizaka 1978). It also appeared that IgE could also bind to other cell types (including B-lymphocytes as well antigen presenting cells like monocytes) but the binding to these cells was less avid than to mast cells and basophils (Spiegelberg & Melewicz 1980;Melewicz et al. 1982;Spiegelberg et al. 1983). The main focus of researchers in the 1980s was to try and identify the receptors involved in the high and lower affinity binding of IgE and also to understand the nature of the binding of IgE by its receptors and the role this played in the effector functions of the various cells that expressed these receptors.

By 1990 the high affinity Fc receptor for IgE (FcεRI) on mast cells and basophils had been identified and it was known that it is expressed as a tetrameric structure (an α, a β and 2 γ subunits) on the membrane of these specific cell types (Blank et al. 1989;Kinet et al. 1991;Metzger 1992a). Research looking into the pivotal role played

by the interaction of IgE with FcεRI in allergic inflammation then focused on several aspects almost concurrently. These included:

a. ***Cellular expression***

Researchers began to look into the possible expression of FcεRI on other cell types besides mast cells and basophils (including antigen presenting cells such as blood monocytes, blood and tissue dendritic cells (e.g. Langerhan's cells) and, most intriguingly in the context of allergic inflammation, blood and tissue eosinophils). When it soon became apparent that high affinity Fc receptors for IgE could indeed be expressed on many of these cell types (Maurer et al. 1994;Maurer et al. 1996;Bieber 1996;Gounni et al. 1994b), this led to further questions such as:

- What is the relative frequency of receptor expression on different cell types?
- Is it always necessary for all three subunits to be expressed on the surface of all these cells?
- Are these receptors expressed constitutively on all these cells or can they be up or down regulated?
- If surface expression of these receptors is inducible, what are the factors that affect this?
- How does the presence of FcεRI on various cell types affect their roles?

Finding the answers to these questions became very much intertwined with other ongoing research into high affinity Fc receptors, elucidated below.

b. ***Structure***

One fundamental question that needed answering was why IgE bound to FcεRI has such a slow dissociation phase (since this was known to be a critical rate limiting step resulting in the interaction between these two molecules having such a uniquely high binding affinity) (Wank et al. 1983;McDonnell, Calvert et al. 2001). Furthermore, what is the basis of the observed 1:1 stoichiometry of binding between IgE and its high affinity receptor (i.e. an individual receptor cannot bind more than one IgE molecule at any given moment) (Robertson 1993;Keown et al. 1997) in contrast to Fc receptors for other immunoglobulin classes that often demonstrate 1:2 stoichiometry? It was hoped that greater understanding of the basic processes involved could, in due course, lead to the development of new therapeutic agents which could disrupt this

interaction. Therefore research focused not just on determining the molecular structures of the three subunits that form the FcεRI complex but also on trying to understand the nature of IgE binding to its receptor. Researchers worked particularly on trying to solve the three dimensional crystal structure of the α-chain of the high affinity receptor (FcεRIα) – the chain on which the binding site for immunoglobulin E is situated (Blank et al. 1991;Hakimi et al. 1990) – as well as that of IgE actually bound to FcεRIα in an effort to try and determine the precise topology of this particular receptor-ligand interaction.

c. ***Function***

As the diversity of cell types expressing FcεRI became apparent, the obvious questions that arose were whether these receptors were functional and, if so, what role they played in different cells. Increasingly this led to an appreciation that the presence of high affinity receptors for IgE on various cell types could greatly expand the rather limited and well defined roles that had been assigned to these cells in our understanding of inflammatory processes at that time. For instance, researchers began to look at the ability of FcεRI on antigen presenting cells (APCs) to greatly facilitate IgE dependent antigen presentation to T-lymphocytes – a concept known as 'antigen focusing' (Bieber 1996). It was demonstrated that the presence of allergen-specific IgE bound to FcεRI on monocytes increased their ability to present allergen to T-lymphocytes almost a thousand fold *in vitro* (Maurer et al. 1995). Even for the two cell types where the presence of FcεRI had long been known about (mast cells and basophils) increasing knowledge led to the realisation that these cells may well have a wider role than just orchestrating immediate hypersensitivity reactions through IgE mediated degranulation. It became apparent that these cells had the potential to be involved in the initiation and maintenance of chronic allergic inflammation because of their ability to synthesise and secrete pro-inflammatory cytokines (e.g. interleukins- 4 and 13) in an IgE-dependent manner (Bradding 1996;Brunner et al. 1993;Schroeder et al. 1994). Perhaps the most controversial observation was that eosinophils, which have been identified as major effector cells in chronic allergic inflammation, may have surface expression of FcεRI (Gounni et al. 1994a). If the presence of functioning IgE receptors on eosinophils in allergic

diseases could indeed be confirmed, then this might provide a potent mechanism for rapid IgE mediated eosinophil degranulation.

d. ***Intracellular events***

The capture of allergens by allergen specific IgE bound to the α-chains of high affinity Fc receptors on the cell surface is only the start of a cascade of signals and events that may eventually lead to the variety of biological consequences mentioned above (Beaven & Metzger 1993). A lot of research was focused on trying to elucidate the sequence of downstream events resulting from the cross linking of receptor bound IgE by a multivalent allergen. It became apparent that the β- and γ- chains of the receptor complex have important roles to play in this biochemical cascade. By 1995, the presence of a particular 26 amino acid sequence motif, called the immunoreceptor tyrosine-based activation motif (ITAM) (Cambier 1995), had been demonstrated on the cytoplasmic C-terminal tails of both chains (*figure 2*). This sequence had been recognised as playing a central role in intracellular signal transduction by a whole host of immune receptors and its presence on these chains in the FcεRI complex provided important evidence that receptor cross-linking by antigen would result in a number of complex but well defined intracellular signalling pathways being activated. This led to a great flurry of activity aimed at determining these signalling event sequences - and indeed the understanding gained of how the FcεRI orchestrates intracellular signalling events resulted in this receptor complex being considered as a paradigm of how other immune receptors function (Sandor & Lynch 1993).

1.4 Receptors For IgE

Immunoglobulin E has a relatively short half life in serum of around 2.5 days to go along with its low serum concentration (Waldmann et al. 1976;Manz et al. 2005) but a large proportion of the body's IgE is located extravascularly (Dreskin et al. 1987). The biological actions dependent on IgE are most commonly manifested when a multivalent antigen cross-links antigen specific IgE bound to the surface membrane of certain immune effector cells.

Within a few years of the identification and characterisation of IgE it had become apparent that two cell types in particular possessed several thousand surface bound IgE molecules per cell – basophils and mast cells (Conroy et al. 1977;Coleman & Godfrey 1981). By the early 1980s it was also apparent that the receptors on these cells bind IgE with similarly high affinity (with an equilibrium association constant, Ka, of $10^9 – 10^{10}$ M^{-1}) (Coleman & Godfrey 1981;Pruzansky & Patterson 1986) and that cross-linking of surface bound IgE on both cell types was an extremely potent stimulator for the rapid release of vasoactive mediators (such as histamine) stored in intracellular granules in these cells (Conroy et al. 1977;Lowman et al. 1988).

It was also found that IgE could also be found bound to other cells, such as monocytes and B-lymphocytes, but the surface density of IgE molecules on these cells was far less than on basophils and mast cells. Furthermore the affinity of IgE binding on these cell types appeared to be much lower than on basophils and mast cells (Ka being approximately 10^7 M^{-1}) (Melewicz et al. 1982;Spiegelberg & Melewicz 1980;Spiegelberg et al. 1983).

By the mid-1980s therefore it was clear that there were two types of Fc receptors for IgE with the high affinity receptor apparently confined to basophils and mast cells. Indeed by the end of that decade the molecular structure and cell surface configurations of both IgE Fc receptors had been determined (Blank et al. 1989;Kikutani et al. 1986).

In the following sections our current knowledge of the structure and function of the high affinity Fc receptor, FcεRI, will be summarised. Whilst the low affinity Fc receptor also has many significant roles in IgE mediated inflammatory responses (including a major regulatory role in the isotype switching of B-lymphocytes towards IgE production), the main focus of this thesis is the high affinity Fc receptor. Therefore the FcεRII will only be discussed relatively briefly as detailed consideration of its structure and functions falls beyond the scope of this dissertation.

1.5 The High Affinity Fc Receptor For IgE

The high affinity Fc receptor is expressed on cell surfaces as a polymeric complex consisting of a combination of three basic subunits – termed the α-, β- and γ-chains (Metzger 1992a). It is a member of the family of multichain immune recognition receptors (MIRRs). This family contains several multichain receptors found on the surface of cells involved in antigen recognition (including the T and B cell receptor complexes and several types Fc receptors for immunoglobulins) (Keegan & Paul 1992). A characteristic feature shared by members of this family is that they all form complexes consisting of antigen or Fc binding chains (containing extracellular domains belonging to the immunoglobulin superfamily) associated with one or more chains which usually contain the ITAM sequence on their cytoplasmic tails and are therefore involved in intracellular signal transduction pathways.

A. B.

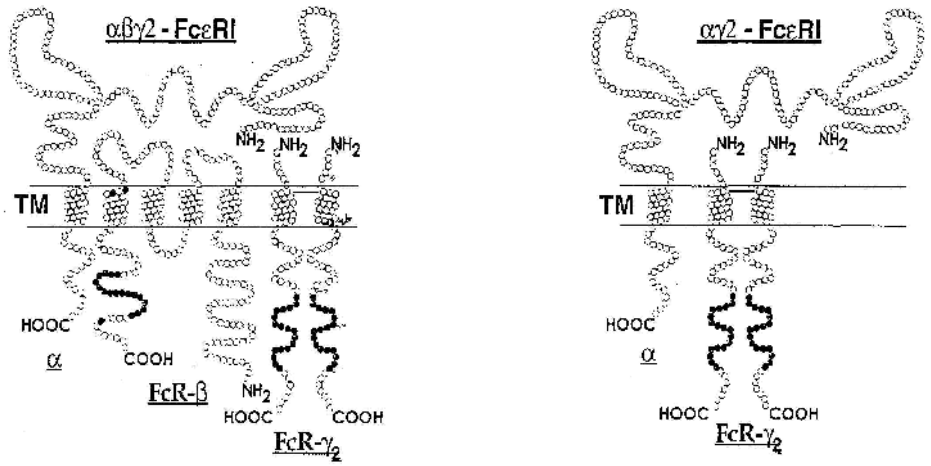

Figure 2: Schematic diagrams of the two different forms of FcεRI present in cell membranes. A) The tetrameric form typically found on mast cells and basophils; B) The trimeric form on antigen presenting cells. The dark circles in the cytoplasmic domains of the β- and γ- chains represent ITAM sequences. TM = transmembrane segments (*from A.M. Scharenberg and J.P. Kinet. 1997. Int Arch Allergy Immunol; 113: 43*)

The α-chain of the FcεRI is a transmembrane protein, with the larger part (containing two immunoglobulin-like domains) situated externally. The γ-chains form a disulphide-linked homodimer with the two identical chains consisting of a short extracellular domain, a relatively longer cytoplasmic tail and a transmembrane

portion. The β-chain is situated mainly within the cell membrane – but transverses it four times so that both the amino- and carboxy- termini are cytoplasmic. Interestingly, and significantly, by the mid-1990s it became apparent that high affinity IgE receptors could be identified on the surface of immune effector cells besides mast cells and basophils (albeit at far lower densities) but that the β-chain is not always necessary for the receptor to be expressed on some of these cell surfaces – in fact it is usually absent on APCs (*figure 2*).

By the early 1990s researchers had been able to isolate complementary DNA (cDNA) coding for all three subunits in humans (Kinet et al. 1987;Kinet et al. 1988;Shimizu et al. 1988;Kuster et al. 1990;Kuster et al. 1992). They were able to use this information to predict the amino acid sequences of these proteins. Furthermore, by transfecting part or whole of the cDNA sequences for the various chains into heterologous mammalian cell lines (e.g. the Chinese hamster ovary line), they were able to study the expression and function of the various components of the FcεRI complex (Kochan et al. 1988;Miller et al. 1989;Kinet et al. 1988;Hakimi et al. 1990). In addition, even though determination of the crystal structure of the receptor (and particularly of the α-chain bound to IgE) was still a few years away, this knowledge of the amino acid sequences allowed very accurate conceptual models of the architecture of the receptor to be developed (Padlan & Helm 1992).

One consequence of the research investigating the structure of FcεRI being done at that stage was the development of a number of well characterised monoclonal antibodies (mAbs) directed at discrete segments of the receptor's component chains (Riske et al. 1991;Rivera et al. 1988). These proved to be useful tools for researchers investigating the expression of this receptor on different cell types – in fact three of the studies described in this dissertation benefited from the availability of receptor specific mAbs.

1.5.1 The α-subunit

The α-chain is the subunit on which the IgE Fc segment binding domain is actually situated (Hakimi et al. 1990;Robertson 1993). It is a 45 kDa glycoprotein with a short

cytosplasmic tail of 33 amino acids (ending with the carboxy terminal) and a 20 amino acid transmembrane domain but with most of the chain situated extracellularly (181 amino acids) (Blank et al. 1989;Garman et al. 1998). The α-chain belongs to the immunoglobulin superfamily.

The immunoglobulin superfamily includes many cell surface or soluble molecules that mediate recognition, adhesion or binding functions in the vertebrate immune system. Members of this superfamily share some structural homology but do not necessarily have similar functions. Although they probably share a common evolutionary pathway, they do not necessarily demonstrate genomic association – indeed their genes are often on different chromosomes. The main characteristics of molecules in this family is that they possess at least one 'immunoglobulin domain' – a region of 70-110 amino acids homologous to the V or C domains on immunoglobulins – with immunoglobulin folds (stabilised by conserved cysteine residues contributing to an intra-chain disulphide bonded loop) (Williams & Barclay 1988).

The amino acid sequences obtained from analysis of cDNA for FcεRIα predicted two disulphide-linked immunoglobulin folds in the extracellular portion of the α-chain (from amino acids positions 51 to 93 and 132 to 176) (Padlan & Helm 1992). This therefore means that the extracellular α-chain comprises of two immunoglobulin domains – which can be termed α(1) and α(2). By around 1993, the main IgE binding region on the FcεRIα had been mapped to the α(2) domain (Mallamaci et al. 1993;Robertson 1993) – but it was also apparent that the presence of structural elements within the α(1) domain are also necessary to impart high affinity IgE binding activity (Mallamaci et al. 1993). By this time similar analytical work had been done on IgE and the FcεRIα binding site had been identified on the two Cε3 domains of the Fc fragment (Basu et al. 1993;Beavil et al. 1993;Presta et al. 1994).

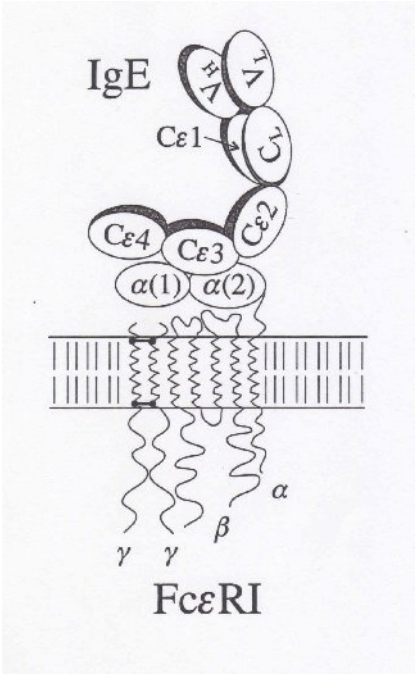

Figure 3: Schematic representation of a tetrameric FcεRI complex showing the two extracellular immunoglobulin domains of the α subunit– α(1) and α(2) – and the IgE binding site on these domains (from B.J. Sutton and H.J. Gould, *Allergy and Allergic Diseases Ed. A. B. Kay*)

The extracellular region of FcεRIα contains seven potential N-linked glycosylation sites which can bind carbohydrate moieties (Garman et al. 1998). In fact attached carbohydrates account for some 30-40% of a receptor's weight (Kanellopoulos et al. 1980;LaCroix & Froese 1993). However several studies have shown that this large carbohydrate presence does not interfere with the ability or the affinity of the receptor to bind IgE – thus indicating that the carbohydrate moieties are not located in the IgE binding site (Pecoud et al. 1981;Blank et al. 1991;Nettleton & Kochan 1995). Other studies would suggest however that glycosylation of the extracellular chain does have significant roles both in the intracellular transport of the chain to the cell surface (by enabling efficient folding of the α-chain in the endoplasmic reticulum) and also at the cell surface by enabling the chain to be correctly orientated (Pecoud et al. 1981;Letourneur et al. 1995).

1.5.1.1 Structural analyses providing insights into the interaction between IgE and the α-subunit of the FcεRI

In 1998 Garman et al finally solved the crystal structure of the extracellular regions of the α chain of the IgE high affinity Fc receptor by using X-ray diffraction at 2.4 Å resolution. They discovered a very acute bend between the two Ig-like domains, α(1) and α(2). The acuteness of this bend is almost unique when compared to other Ig

domain structures and it helps produce a large convex surface within the α(2) domain (contiguous with the α(1)α(2) interface) that is involved in binding the IgE Fc fragment. Adjacent to this area is a prominent loop extending from the receptor surface. This loop is formed by an unusual arrangement of four exposed tryptophan molecules and forms a complementary contact surface for IgE, thereby conferring specificity for IgE (Garman et al. 1998). This analysis also confirmed that none of the seven densely glycosylated regions impinge on the IgE binding site.

Within the next four years two further high resolution x-ray diffraction analyses were published – in 2000, Garman et al went on to elucidate the structure of the Fc fragment of IgE when it is actually bound to the α-chain of FcεRI (Garman et al. 2000) and in 2002 Wan et al published the detailed crystal structure of the Fc fragment of IgE (Wan et al. 2002). These studies finally helped provide feasible answers to two fundamental questions about the nature of this interaction – the reason for the unique 1:1 stoichiometry and the basis of the exceptionally slow dissociation rate when the two molecules actually interact.

The crystal structure of the Fc fragment determined by Wan et al demonstrated that its pair of Cε2 domains form a distinctive disulphide-linked immunoglobulin domain interface and are folded back asymmetrically onto the Cε3 and Cε4 domains. This causes an acute bend in the IgE molecule, resulting in a compact, tightly bent conformation (just as had previously been described by Zheng et al. in 1991). The structural implication is that there is a substantial conformational change involving this Cε2 hinge when the IgE molecule binds to its Fc receptor, FcεRI, which makes it very difficult for the molecules to prise themselves apart leading to the exceptionally slow dissociation rate. However this Cε2 region may also allow bending of the antibody when attached to its receptor and thus allow the Fab domains to be optimally positioned to capture multivalent antigens (Wan et al. 2002).

The crystal analysis of the IgE-Fc–FcεRIα complex (Garman et al. 2000) revealed two interactions site with the two Cε3 domains of the Fc fragment binding asymmetrically two distinct sites on the α-chain: one to the convex Fc binding surface formed in the α(2) domain and the other to the previously described loop

formed by the four exposed tryptophan molecules. This dual binding of both Cε3 domains precludes further binding of the IgE molecule to any other Fc receptor – resulting in 1:1 binding only.

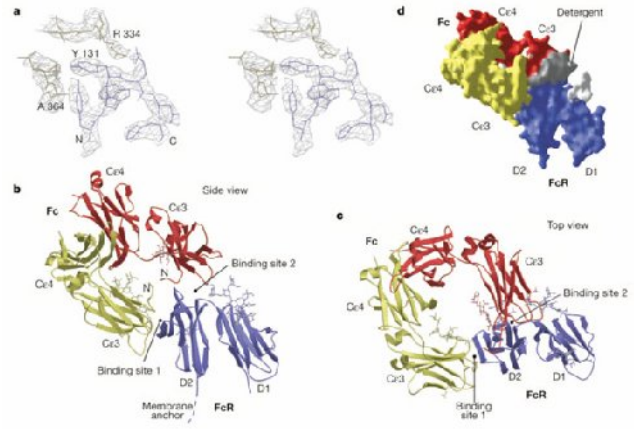

Figure 4: Overview of the crystal structure of the IgE-Fc/FcεRIα complex showing the dual IgE Cε3 binding sites on domain 2 (D2) of the FcεRIα chain (which is depicted in blue) (from Garman et al. 2000).

Cross-linking of cell surface receptor bound IgE by multivalent antigens can induce a number of effector responses, depending on the nature of the cell. In mast cells, for instance, this surface binding of antigen can result in rapid secretion of preformed, granule stored mediators (such as histamine) and also initiate synthesis and subsequent release of lipid mediators and cytokines (Lowman et al. 1988;Toru et al. 1998). In antigen presenting cells, on the other hand, the receptor bound antigen may be internalised and processed for presentation to other effector cells such as T cells (Bieber 1996;Stingl & Maurer 1997). However the α-chain has no significant signalling capacity and is therefore dependant on the availability one or more of it associated subunits (β- or γ-chains) to set off well defined cascades of intracellular signal leading to cell activation. Although neither of these chains possesses intrinsic catalytic activity, both contain the ITAM sequence and are closely associated with cytoplasmic protein tyrosine kinases. Their roles in signal transduction are elucidated in section 1.5.3.

1.5.2 The β-subunit and γ-subunits – structure, function and cellular expression

In the decade following the first description of the complete structure of the human FcεRI by Blank and his colleagues in 1989 (Blank et al. 1989), huge strides were

made in defining the structure and function of the two other chains involved in the receptor complex.

The γ-chains are transmembranic polypeptides with each chain having a molecular weight of 7– 9 kDa. They exist as a homodimer which is actually identical to the γ-subunit of FcγRIII (CD16 – one of the low affinity Fc receptors for IgG) (Letourneur et al. 1991). The γ-chain complex is also found associated with other Fc receptors, including FcγRI (Ernst et al. 1993;Scholl & Geha 1993) and FcαRI (Pfefferkorn & Yeaman 1994), on cells expressing these receptors. FcεRIγ is also structurally closely related to the ζ and η chains of the T cell receptor (TCR) complex – for instance each γ-chain has 55% structural homology with the ζ chain (Kuster et al. 1990;Rodewald et al. 1991). In fact substitution of ζ chains in a TCR and FcεRIγ by one another can still result in functional receptor complexes (Howard et al. 1990;Rodewald et al. 1991).

The β-chain has an unusual orientation as it traverses the cell membrane four times with both the C- and N- termini being on the intracellular side of the membrane (Kinet et al. 1988) (*figure 2*). It is a protein around 245 amino acids in length with a molecular weight of 26.5 kDa (Kuster et al. 1992). By the early 1990s it had also become apparent that whilst the β-chain appeared to be a constitutive component of the FcεRI on mast cells and basophils, there may be a naturally occurring variant of FcεRI without the β-chain. This possibility was first suggested by experiments involving co-transfection of genes for the three different FcεRI chains into cell lines and then inducing surface expression of the proteins. It was found that whilst the α- and γ-chains were always necessary for the receptor to be expressed on the cell surface, stable surface expression could be achieved without the presence of a β-chain (Miller et al. 1989;Kinet et al. 1991). It came as no surprise therefore when researchers soon found that FcεRI expressed on cell types other than mast cells or basophils are usually of the $\alpha\gamma_2$ variety (i.e. they lack the β-chain) (Bieber 1996).

The ITAM is present in both β- and γ-chains but differs slightly in both chains. The ITAM in the β-chain has a shorter segment between its first two tyrosines and in

addition has a third tyrosine which is absent in the γ-chains (Nadler et al. 2001). Furthermore the cytoplasmic protein tyrosine kinase Lyn (a member of the Src family of tyrosine kinases) is found closely, and preferentially, associated with the β-chain (Jouvin et al. 1994). In contrast the γ-chain ITAM tends to associate with the tyrosine kinase Syk (Jouvin et al. 1994). This differing pattern of tyrosine kinase association is believed to help explain an important difference in the roles of the two chains in signal transduction. The γ-chain appears to be the primary chain responsible for transmitting cell activation signals (Letourneur & Klausner 1991;Wilson et al. 1995). In contrast the β-chain does not have autonomous cell activation capability but is an excellent amplifier of γ-chain mediated activation signals with a gain of five to seven fold (Lin et al. 1996;Scharenberg & Kinet 1997). Thus the two chains act synergistically when the tetrameric variant of FcεRI is present (e.g. on mast cells) to provide a larger amplitude activation signal then produced by the trimeric complex on antigen presenting cells. This amplifier property of the β-chain appears to be almost unique amongst the known component chains of MIRRs.

1.5.3 A brief summary of signal transduction cascades initiated by FcεRI

The FcεRI has proved to be a paradigm of immune receptor initiated signalling events. The study of intracellular signalling pathways is a highly specialised field but the complex signal transduction cascades generated by the FcεRI are quite well understood by now and have been extensively reviewed elsewhere (Beaven & Metzger 1993;Beaven & Baumgartner 1996;Nadler et al. 2001). A detailed discussion of these events is beyond the scope of this dissertation. However some understanding of the sequence of events from antigen binding to cell activation is fundamental to appreciating the important role of this receptor in cells involved in allergic inflammation. Therefore a relatively brief description of the main events involved follows.

Multivalent antigens cross-linking IgE molecules bound to the α-chains on FcεRI in the cell membrane cause the rapid aggregation of these receptors within a couple of seconds (Metzger 1992b). The next step is the phosphorylation of tyrosine residues in the intracellular tails of the receptor chains. The presence of the tyrosine kinase Lyn

is crucial for this process and, as has been described above, this is closely associated with the β-chain. As a consequence of the tight receptor aggregation Lyn kinases do not just phosphorylate β-chain tyrosine molecules, but are also able to transphosphorylate tyrosine residues in adjacent γ-chains. The associated Syk kinases also bind to these phosphorylated ITAMs and, significantly, Syk also becomes phosphorylated by Lyn. Phosphorylated Syk is much more active than unphosphorylated Syk – thus the presence of the β-chain and its close association with Lyn provides a great boost to the signals generated by γ-chains. These phosphorylation events are also very rapid and are complete within 5 to 15 seconds of receptor cross-linkage (Pribluda & Metzger 1992;Suzuki et al. 1997). The signalling cascade then moves on from these receptor associated processes to a number of signalling pathways that are present within the cell. These usually involve either activation of other enzymes with resultant effects on cytoplasmic substrates or activation of 'second messengers' including activated signalling proteins and, particularly important, increased concentrations of intracellular calcium ion concentrations (Beaven & Baumgartner 1996).

Although these signal pathways are present in most cells, the upstream signals generated by the receptor complex result in well defined effector responses which all happen within particular time frames. For instance migration of granules to the cell surface and subsequent exocytosis of preformed mediators such as histamine happens within seconds. Another fairly rapid sequence of signals results in the activation of phospholipases (such as PLA_2, PLC, PLD). One consequence of these is the synthesis of the arachidonic acid derived mediators leukotrienes (this process takes several minutes) (Beaven & Metzger 1993). Another consequence is the activation of cytokine genes in the nucleus, transcription of these genes and production and secretion of these cytokines. This process can take several hours – although the release of inflammatory cytokines could be greatly speeded up if preformed cytokines were able to be stored in granules (a possibility that was investigated in one of the constituent papers of this thesis).

1.6 The Low Affinity Fc Receptor For IgE

There is a second type of cell membrane receptor for IgE that has binds the Fc portion of IgE with an affinity almost two orders of magnitude less than FcεRI (with a Ka of 10^7 M^{-1}). In contrast to all other known immunoglobulin receptors, the low affinity Fc receptor for IgE (FcεRII or CD23) does not belong to the immunoglobulin superfamily. It is actually a C-type lectin (Bonnefoy et al. 1993a). Lectins are usually defined by their ability to bind carbohydrates in glycoproteins but FcεRII binds IgE in a Ca^{2+} dependent manner that does not in fact involve binding to the carbohydrate moieties of IgE (Vercelli et al. 1989a).

IgE is not the only natural ligand for CD23. It binds to the glycosylated cell surface molecule CD21 (also known as complement receptor 2, CR2) (Aubry et al. 1992;Bonnefoy et al. 1993b), as well as the CD11b and CD11c components of integrin receptor complexes (Lecoanet-Henchoz et al. 1995). As might be expected from this variety of natural ligands CD23 shows great functional diversity.

CD23 molecules were originally described on B-lymphocytes where, interestingly, they appear to be closely associated with HLA-DR molecules on the cell surface (Bonnefoy et al. 1988). There appear to be two membrane bound isoforms – CD23a is expressed constitutively on B-cells whereas CD23b expression is inducible by IL-4 (and inhibited by IFN-γ) on B-cells and several other immune effector cells, including macrophages, Langerhans cells, follicular dendritic cells, eosinophils and T-lymphocytes (Yokota et al. 1988;Bonnefoy et al. 1993a;Lee et al. 1993). Significantly, CD23 molecules unoccupied by IgE can be cleaved off from cell surfaces by proteases such as metalloproteinase ADAM 10 (Hibbert et al. 2005; Lemieux et al. 2007).

CD23 molecules expressed on B-lymphocytes appear to have several functions which may be important in the IgE mediated inflammatory cascade:

- Binding of IgE / allergen complexes to membrane bound FcεRII molecules greatly enhances the antigen-presenting capacity of B-lymphocytes to T-lymphocytes (Pirron et al. 1990;van der Heidjen et al. 1993).

- Membrane bound CD23 molecules, by ligating CD21 molecules on T-lymphocytes, may also have a role in stabilising T- and B-cell cognate interactions (Bonnefoy et al. 1993b).
- Both of these actions may help promote more efficient allergen specific T-lymphocyte responses (Getahun et al. 2005;van Neerven et al. 2006).
- CD23 facilitated IgE-mediated allergen presentation by B-cells has been demonstrated to help promote T_H2 type responses *in vitro* (van der Heijden et al. 1993). In an allergen challenge mouse model, it was shown that an anti-CD23 mAb could also effectively inhibit production of the archetypical T_H2 cytokines IL-4 and IL-5 *in vivo* (Coyle et al. 1996).
- Membrane bound and soluble CD23 also appear to play important roles in regulating IgE synthesis. For instance, CD23 facilitated allergen presentation on B-cells significantly up-regulates allergen specific antibody synthesis – interestingly of IgM and IgG classes as well as IgE (Heyman 2002). Soluble CD23 also appears to play an important role in up-regulating IgE synthesis, possibly by coligating cell membrane IgE and CD21 (Hibbert et al. 2005).
- On the other hand, coligation of membrane CD23 and IgE molecules seems to down regulate IgE synthesis (Hibbert et al. 2005).

The possibility of an intriguing natural regulatory feedback loop is raised by the observations described above that CD23 may play a role in regulating T_H2 responses since the T_H2 cytokine IL-4 is of course not just important for CD23 synthesis but is also vital for the isotype switching of B-cells towards IgE production (Vercelli et al. 1989b;Aubry et al. 1992).

1.7 Introduction to the four core papers of this thesis

Four papers are presented and discussed in the following chapters. All of these papers involved research into aspects of blood derived FcεRI bearing cells. The title of each paper and a brief summary of the nature of the study described are given below.

1.7.1 Expression of high affinity IgE receptors (FcεRI) on peripheral blood basophils, monocytes and eosinophils in atopic and non-atopic subjects: Relationship to serum immunoglobulin E concentrations (Sihra et al. 1997a).

As has already been described above, by the mid 1990s it was becoming evident that cell types other than basophils and mast cells may have some degree of FcεRI expression. Expression of this receptor had been reported on some peripheral blood monocytes from both atopic and non-atopic subjects (Maurer et al. 1994;Reischl et al. 1996). Intringuingly, there were also some reports of FcεRI expression on blood eosinophils from subjects with hypereosinophilic syndromes as well as some tissue eosinophils at sites of allergic inflammation (Gounni et al. 1994a;Gounni et al 1994b;Humbert et al. 1996). Several fundamental questions remained unanswered including:

- What is the comparative receptor expression on these three cell types in individuals?
- Do blood eosinophils really express FcεRI?
- Is there increased receptor expression in any, or all, of these three leucocytes in subjects with atopic diseases compared to non-atopic individuals?
- If so is this difference due to the particular atopic disorder (e.g. asthma, allergic rhinitis or atopic dermatitis) or, as had been demonstrated on basophils (by indirect methods some two decades previously), does receptor expression on these cell correlate positively with serum IgE?

Answers to these basic questions were necessary to begin to fully appreciate the diversity of roles the high affinity IgE Fc receptor could play in allergic disorders, but previously it had been difficult to perform such a study for the following technological reasons:

1. At that time there were no basophil specific markers which allowed identification of unseparated basophils in blood.
2. Monoclonal antibodies (mAbs) specific for the various components of the FcεRI complex had only just been developed.
3. The technology of multi colour flow cytometry and cell sorting (which was used in this study) had only just become widely available. This is a powerful

and very sensitive tool which can rapidly assess thousands of events in solution (e.g. cells and/or molecular complexes etc) and accurately identify the events of interest even if they are just at single figure level.

In the paper presented in Chapter 2, we were able to use dual colour flow cytometry to evaluate FcεRIα expression on unseparated peripheral blood mononuclear cells (PBMCs) and get a 'side-by-side' comparison of expression on all three cell types. In this study we also took advantage of two characteristic properties of basophils – their uniquely high level of constitutive FcεRI expression and the characteristic way they scatter light during flow cytometric analysis – to develop a novel way of identifying basophils in blood without having to purify them.

1.7.2 Unstimulated peripheral blood basophils in atopic and non-atopic subjects express intracellular interleukin-4: detection by flow cytometry (Kon, Sihra et al. 1998).

The second paper (in Chapter 3) made further use of the novel flow cytometric method of identifying unseparated basophils in peripheral blood described above to examine their potential to rapidly secrete stored cytokines (in particular interleukin-4). Previously studies on purified basophils obtained from atopic subjects had demonstrated their capacity to secrete IL-4 after *in vitro* IgE stimulation (Brunner etal. 1993;Schroeder et al. 1994).

In this study, multi-colour flow cytometry was used to analyse whether unseparated basophils in PBMCs from atopic and non-atopic donors constitutively express intracellular IL-4 which could then potentially be released following FcεRI cross-linkage.

1.7.3 The effect of cyclosporin A on the allergen-induced late asthmatic reaction (Sihra et al. 1997b).

The immunosuppressive agent cyclosporin A (CsA) had been shown both to reduce disease exacerbations and improve lung function in patients with severe

glucocorticoid-dependent asthma (Alexander et al. 1992) and also act as a steroid sparing agent in a similar group of patients (Lock et al. 1996). Whilst it was initially thought that its efficacy resulted mainly from its inhibitory effects on the secretion of pro-inflammatory cytokines from activated T-lymphocytes, evidence was accumulating that CsA has other actions which could also have therapeutic benefit in asthma. In particular it had been shown to rapidly and potently inhibit mediator release from FcεRI bearing cells (mast cells and basophils) (Casolaro et al. 1993;Triggiani et al. 1989).

This paper describes a randomised, double blind, placebo controlled cross-over study that evaluated the effects of pre-treatment with CsA on allergen induced early and late asthmatic responses (EAR and LAR, respectively). The EAR is thought to result mainly to result from the rapid release of pre-formed histamine and newly generated lipid mediators (such as cysteinyl leukotrienes) from FcεRI bearing cells (mainly mast cells, possibly tissue basophils) following cross linkage of allergen specific IgE bound to the FcεRI (Casale et al. 1987;Liu et al. 1991). In contrast, the LAR features a characteristic inflammatory response which particularly involves T-lymphocyte activation, increased production of 'eosinophil-active' cytokines and accumulation of eosinophils in the bronchial mucosa (Bentley et al. 1997). The study described in Chapter 4 attempted an *in vivo* 'dissection' of the various possible mechanisms of CsA in asthma and tried to provide an answer to the question whether the observed inhibitory actions on FcεRI bearing cells *in vitro* are indeed useful in this model of allergen induced asthma.

1.7.4 Effects of maintenance or cessation of prolonged allergen specific subcutaneous injection immunotherapy on humoral immune responses and cell surface IgE receptor expression in peripheral blood (Sihra et al. 2000).

The efficacy of subcutaneous allergen specific immunotherapy (SCIT) for grass pollen seasonal allergic rhinitis is now well established – and a series of studies led by Professor Stephen Durham have been influential both in establishing its efficacy and also in elucidating the immunomodulatory effects of SCIT (Varney et al. 1991;Varney et al. 1993;Walker et al. 1995;Durham et al. 1996;Durham et al. 1999).

A major change during SCIT treatment appeared to be a change in allergen specific cytokine responses from T_H2 to T_H1 type (Till 1997). However during SCIT treatment there are other observed changes in peripheral blood (Durham & Till 1998;Till et al. 2004), including:

- attenuation of seasonal elevations in serum levels of allergen specific IgE;
- gradual decline in specific IgE levels;
- increased concentrations of allergen specific IgG antibodies, so called 'blocking antibodies';
- decreased basophil reactivity *in vitro* to IgE dependent stimuli, presumed to be reflected *in vivo* by reduced amounts of mast cell or basophil derived mediators in nasal secretions, has also been described.

In a seminal study by Professor Durham (Durham et al. 1999), 27 subjects with seasonal allergic who had received continuous grass pollen SCIT for 3-4 years were randomised, in blinded manner, either to continue receiving active IT or placebo injections for a further 3 years. It was found that there was no difference in symptom scores, medication use and cutaneous late phase responses between both groups at the end of this study period. Notably, improvements in all these parameters compared to baseline values had been maintained 6-7 years after initiation of SCIT even in the group who had not received active IT for 3 years. Furthermore there was evidence of persistence of the skewed cytokine profile from T_H2 to T_H1, with reduced numbers of IL-4 mRNA+ cells following cutaneous allergen challenge (with no difference between the two treatment groups) at the end of the study. Questions that remained to be answered included whether the humoral immunological changes associated with successful immunotherapy described above also persisted in these two groups of patients who had received 3 years of allergen specific IT whether or not they had continued to receive prolonged SCIT. Furthermore, is prolonged IT associated with changes in IgE receptor expression (both high and low affinity) on blood cells?

In the study described in the final paper of this thesis (Chapter 5), blood samples were obtained before and during the final pollen season of the study from all these 27 patients as well as 2 control groups (one non atopic group and one group of subjects with seasonal allergic rhinitis who had never received SCIT) and comparative

analyses were performed for serum total IgE (immunoglobulin E) concentrations, serum concentrations of allergen *(Phleum pratense)* specific IgE and IgG, FcεRI expression on basophils and monocytes and CD23 (the lower affinity receptor for IgE) expression on B-lymphocytes and serum concentrations of the soluble form of FcεRIα.

Chapter 2

Cells Expressing FcεRI In Blood

2.1 Expression Of High-Affinity IgE Receptors (FcεRI) On Peripheral Blood Basophils, Monocytes, And Eosinophils In Atopic And Non-Atopic Subjects: Relationship To Total Serum IgE Concentrations

(published in the Journal of Allergy and Clinical Immunology 1997; 99: 699-706)

Expression of high-affinity IgE receptors (FcεRI) on peripheral blood basophils, monocytes, and eosinophils in atopic and nonatopic subjects: Relationship to total serum IgE concentrations

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Background: High-affinity IgE receptors (FcεRI) have been identified on peripheral blood basophils, monocytes, and eosinophils; but the relative receptor expression on these cells and their relationship to atopy are unclear.
Objective: The aim of this study was to compare FcεRI expression on these cell types and assess their relationship to total serum IgE concentrations in subjects with atopic asthma, rhinitis, or dermatitis compared with nonatopic control subjects.
Methods: Flow cytometry was used to evaluate FcεRI expression by determining the specific mean fluorescence of the binding of two anti-FcεRI α-chain monoclonal antibodies (15-1, which competes with IgE for receptor binding, and 22E7, which is noncompetitive).
Results: Compared with basophils FcεRI expression (determined by 22E7 specific mean fluorescence) was greatly reduced on monocytes and was only detectable on eosinophils in a small minority of subjects. Nevertheless, FcεRI expression on all three cell types was significantly increased in atopic patients compared with nonatopic control subjects ($p < 0.0001$ for basophils, $p = 0.003$ for monocytes, and $p = 0.039$ for eosinophils). FcεRI expression on both basophils and monocytes in all subjects correlated significantly with serum IgE concentrations ($r = 0.86$ and 0.55, respectively; $p < 0.001$). For each subject, and on all three cell types, the specific mean fluorescence after 22E7 staining was greater than with 15-1, implying some degree of receptor occupancy.
Conclusion: FcεRI expression on peripheral blood monocytes was considerably less than on basophils and barely detectable on eosinophils. Elevated FcεRI expression was observed in atopic subjects with all three cell types, suggesting a role for these receptors in IgE-mediated allergic inflammation. The possibility of common regulatory mechanisms was suggested by the correlation of FcεRI expression on basophils and monocytes with serum IgE concentrations. (J Allergy Clin Immunol 1997;99:699-706.)

Key words: IgE receptor (high affinity), atopy, IgE, basophils, monocytes, eosinophils

Abbreviations used

AA:	Atopic asthma
AD:	Atopic dermatitis
AR:	Atopic rhinitis
FITC:	Fluorescein isothiocyanate
FcεRI:	High-affinity IgE receptor
mAb:	Monoclonal antibody
mfi:	Mean fluorescence intensity
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
PE:	Phycoerythrin
smf:	Specific mean fluorescence

The presence of cell membrane receptors specific for the Fc region of immunoglobulins provides a mechanism for coupling antibody-antigen interactions with cellular effector responses. FcεRI, the high-affinity receptor for the Fc region of IgE, is a tetrameric complex comprised of an α-subunit, a β-subunit, and a dimeric γ-subunit. The heavily glycosylated α-chain binds monomeric IgE with an affinity almost two orders of magnitude greater than FcεRII, the other cell surface Fc receptor for IgE (the equilibrium association constants, K_a, are 10^9 M^{-1} and 10^7 M^{-1}, respectively).[1, 2] The β- and γ-subunits are required for stabilization of transmembrane insertion of the α-chain and for signal transduction.[3, 4]

Until recently FcεRI expression in human beings was believed to be confined to basophils and mast cells. The FcεRI has now been identified on epidermal Langerhans cells[5, 6] and blood monocytes in both atopic and normal subjects.[7, 8] The receptor has also been described on blood eosinophils in patients with hypereosinophilic syndromes,[9] on eosinophils in skin biopsy specimens from patients with atopic dermatitis and bullous pemphigoid,[10] and on a small percentage of eosinophils from bronchial biopsy specimens from patients with atopic and nonatopic asthma.[11] However, there is still uncertainty regarding the relative expression of FcεRI between different cell types and the relationship of these receptors to serum IgE concentrations in atopic disease.

It has previously been demonstrated by indirect methods that the numbers of receptors on basophils that bind IgE with high affinity correlated with serum IgE concentrations in vivo.[12] We hypothesized that the numbers of

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TABLE I. Clinical details of the four subject groups

Subject group	Age (yr)		M:F	PC_{20} methacholine	No. of subjects with positive RAST results (>0.7 IU/ml)	Total IgE (IU/ml)		Eosinophils ($\times 10^9$/L)	
	Median	Range				Median	Range	Median	Range
AA (n = 10)	33	26-54	4:6	0.5-3.6	HDM 7 GP 6 Cat 6 Dog 0	245	68-544	0.2	0.1-0.4
AR (n = 10)	30.5	22-47	8:2	All >16	HDM 9 GP 6 Cat 1 Dog 1	177	51-644	0.2	0.1-0.4
AD (n = 8)	30	20-41	5:3	Not done	HDM 6 GP 7 Cat 6 Dog 4	2073	373-35,700	0.5	0.1-1.4
NC (n = 10)	27	23-33	2:8	All >16	HDM 0 GP 0 Cat 0 Dog 0	18	4-35	0.1	0.1-0.5

Serum IgE comparisons: $p < 0.001$ for each of AA, AR, and AD versus NC; $p < 0.01$ for AA and AR versus AD; $p = 0.57$ for AA versus AR.
HDM, House dust mite; *GP,* grass pollen, *NC,* normal control.

FcεRI on peripheral blood monocytes and eosinophils are elevated in atopic subjects compared with normal subjects, and as with basophils, receptor expression can be correlated with circulating IgE concentrations. We further hypothesized that this relationship exists irrespective of the nature of the coexisting atopic disease.

We have therefore performed dual color flow cytometry, using two fully characterized murine monoclonal antibodies (mAbs) specific for the α-chain of human FcεRI to evaluate receptor expression on peripheral blood basophils, monocytes, and eosinophils in atopic and nonatopic subjects and to determine whether there was any difference in FcεRI expression on these cells in different atopic diseases—specifically asthma, rhinitis, and dermatitis. We have also investigated the relationship between serum IgE concentrations and FcεRI on basophils and monocytes. One of the mAbs used, 15-1, competes with IgE for binding to FcεRI-α and therefore only binds to receptors unoccupied by IgE,[5] whereas the second mAb, 22E7, is noncompetitive[13] and therefore binds to all FcεRI, whatever their occupancy status.

METHODS

Subjects

Thirty-eight volunteers were studied: 10 patients with atopic asthma (AA), 10 subjects with atopic rhinitis (AR), eight subjects with widespread chronic atopic dermatitis (AD), and 10 healthy nonatopic subjects (control group).

Atopy was defined as either a positive skin prick test response (wheal diameter >3 mm compared with diluent control) or a positive RAST response (>0.70 IU/ml) to extracts of one or more of a panel of common aeroallergens (including house dust mite, mixed grass pollen, mixed tree pollen, mixed molds, and cat and dog fur). Patients with asthma all gave a clear history consistent with asthma and had current symptoms and a PC_{20} methacholine of less than 6 mg/ml with greater than 20% FEV_1 reversibility, either spontaneously or after inhalation of β-agonist. Patients with AR had no evidence of asthma and were atopic (as defined above) but gave a clear history of seasonal or perennial rhinitis. PC_{20} methacholine was more than 16 mg/ml for all the subjects in this group. Patients in the AD group met accepted diagnostic criteria,[14, 15] with dermatitis being their dominant atopic disorder, although all had manifestations of other allergic disorders (Table I). Normal control subjects had no clinical or laboratory evidence of atopy or asthma. Subjects who had received oral corticosteroids within the preceding 3 months or immunotherapy during the preceding 12 months were excluded from the study. Table I contains full details of all four subject groups.

Subjects in the AA, AR, and normal control groups were recruited either from among patients attending the allergy clinic or staff members of the Royal Brompton Hospital, London. The subjects with chronic AD attended the Dermatology Clinic at the Chelsea and Westminster Hospital, London. The study was approved by the Ethics Committee of the Royal Brompton Hospital.

Monoclonal antibodies (mAbs)

Monoclonal antibody 15-1 (isotype IgG_1) was a kind gift from Dr. J-P. Kinet, Molecular Allergy and Immunology Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Rockville, Md.). Monoclonal antibody 22E7 (isotype IgG_1) was a kind gift from Drs. R. Chizzonite and J. Kochan, Hoffman–La Roche Inc. (Nutley, N.J.). Murine IgG_1 isotype control antibody, phycoerythrin (PE)-conjugated goat anti-murine IgG_1 mAb and fluorescein isothiocyanate (FITC)–conjugated anti-human CD14 (mouse IgG_1) were obtained from Caltag Laboratories (San Francisco, Calif.). FITC-conjugated anti-human CD9 (mouse IgG_1) was obtained from Sera Lab Ltd. (Crawley Down, Sussex, U.K.).

Dose-response experiments to establish saturating concentrations of both the anti-FcεRI antibodies for detection by flow cytometry were performed on both peripheral blood mononuclear cells (PBMCs) and eosinophils (identified by granulocytes

Current atopic disease (no. of subjects)	Concurrent treatment
Asthma 10	Bronchodilator 10
Seasonal rhinitis 7	Inhaled steroid 8
Perennial rhinitis 3	Nasal steroid 2
Eczema 0	Antihistamine 0
Asthma 0	Bronchodilator 0
Seasonal rhinitis 9	Inhaled steroid 0
Perennial rhinitis 8	Nasal steroid 0
Eczema 0	Antihistamine 2
Asthma 6	Bronchodilator 5
Seasonal rhinitis 2	Inhaled steroid 4
Perennial rhinitis 0	Nasal steroid 1
Eczema 8	Topical steroid 6
	Antihistamine 4
Asthma 0	Nil
Seasonal rhinitis 0	
Perennial rhinitis 0	
Eczema 0	

expressing a characteristic side-scatter vs CD9-staining pattern) before this study began. For both mAbs, the saturating concentration for monocytes and basophils was 3 μg/ml (per 1×10^6 total PBMCs) and for eosinophils an average of 30 μg/ml (per 1×10^6 eosinophils).

Study design

Peripheral venesection was performed on each subject, and 20 to 25 ml of blood was collected in a heparinized syringe. At the same time blood was also collected for RASTs and quantification of total serum IgE concentrations by the CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Five milliliters of 6% dextran in Hanks' balanced salt solution was added to the heparinized blood, and erythrocytes were allowed to sediment. The buffy layer was then collected and mixed with an equal volume of RPMI-1640 medium (Sigma-Aldrich Company Ltd., Poole, Dorset, U.K.) before it was overlaid onto Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 20 minutes at 20° C and 1000 *g*. Cells at the medium-Ficoll interface (PBMCs containing basophils, as well as lymphocytes and monocytes) were collected and processed separately from the cell pellet (containing granulocytes and any remaining erythrocytes). Both cell samples were washed in phosphate-buffered saline (PBS) before the erythrocytes in the granulocyte pellet were lysed by using 10% B.D. lysis solution (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). All further processing of the samples was performed at 4° C.

The PBMC sample concentration was adjusted to 1×10^6 cells in 100 μl of PBS. Twenty microliters of the appropriate primary mAb (20 μg/ml 22E7, 15-1, or isotype control in PBS containing 10% vol/vol human serum) was added (giving a final antibody concentration of 3 μg/ml). After a 30-minute incubation period, the samples were washed twice in PAB (PBS containing 0.5% wt/vol bovine serum albumin and 0.1% wt/vol sodium azide) and then stained with the secondary mAb (goat anti-murine IgG_1 conjugated to PE) diluted 1:40 with 10% human serum in PBS. A further 30-minute incubation period was followed by two washes with PAB and a final 15-minute incubation with 5 μl of anti-CD14 FITC. Granulocytes were treated almost identically except that the final concentration of primary mAb was 30 μg/ml per granulocyte sample containing 1×10^6 eosinophils, and the final stain was with 5 μl anti-CD9 FITC. After final washes, the samples were ready for flow cytometry.

Flow cytometry

The samples were analyzed on a Coulter Epics Elite ESP flow cytometer and cell sorter (Coulter Corp., Hialeah, Fla.). Identical gain settings were used for each sample, and the cytometer flow cell was fully aligned and calibrated with Immuno-Check fluorospheres (Coulter) before each run. This allowed direct and valid inter- and intraindividual comparisons.

From early experiments, it became clear that every subject studied (normal and atopic) had a distinct population of cells (about 2% to 6% of PBMCs) that stained brightly with both anti-FcεRI-α antibodies and had a characteristic side-scatter pattern between lymphocytes and monocytes. This group of cells did not express surface markers such as CD3, CD14, and CD19, which identify other cell types within PBMC samples. With 22E7 staining, this cell population was sorted to 97% ± 1% purity, and cytospins of the sorted cells were made. Staining with May-Grünwald-Giemsa stains confirmed that all these cells were basophils. This very specific method of positively detecting basophils in blood by flow cytometry, which was used for this study, had many similarities to a previously described method, which used side-scatter and surface-bound IgE to identify basophils.[16]

Monocytes were identified by their characteristic CD14 versus side-scatter staining pattern: cell sorting confirmed that cells with this pattern were more than 97% pure monocytes. Similarly, eosinophils were identified by their characteristic CD9 versus side-scatter pattern within the granulocyte sample. Data from 10,000 monocytes and eosinophils were collected and analyzed.

Cellular expression of FcεRI was quantified by determining the specific mean fluorescence (smf). This was the difference between the mean fluorescence intensity (mfi) of cells stained with 22E7 or 15-1 and the mfi of these cells stained with an isotype-identical irrelevant control antibody. Because saturating concentrations of the primary antibody and the same secondary antibody were used, this figure was proportional either to the mean numbers of surface FcεRI per cell (22E7) or mean numbers of FcεRI unoccupied by IgE per cell (15-1).

Although only single samples stained with each antibody were analyzed for each subject, the reproducibility of cell staining was assessed by staining eosinophils from one subject in quadruplicate with 22E7 or the isotype control mAb and determining the coefficients of variation of mfis of these replicate samples.

Statistical analysis

Data were analyzed by using a statistical package (Minitab for Windows, Minitab Release 9.2; Minitab Inc., State College, Pa.). Nonparametric analytical tests were used: Kruskal-Wallis ANOVAR analysis was used to assess variability within the study population; and if this was significant, the Mann-Whitney U test was used to analyze intergroup differences. Correlation coefficients were calculated by Spearman's method with correction for tied values. For all tests, p values less than 0.05 were considered significant.

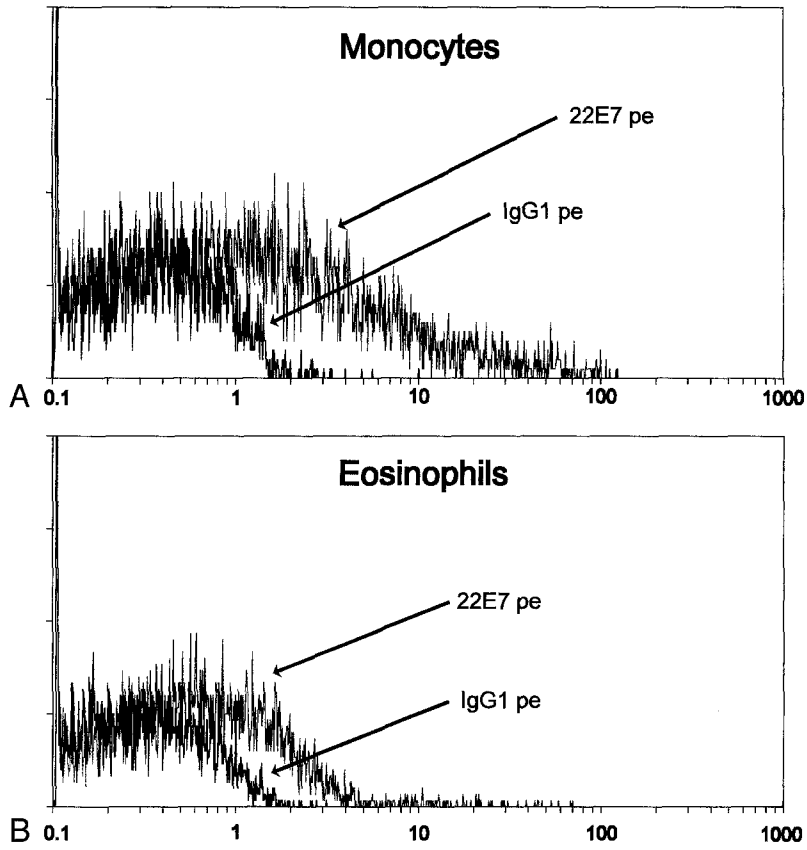

FIG. 1. Representative flow cytometric histograms of monocytes **(A)** and eosinophils **(B)** (from two separate atopic individuals) showing fluorescence detected after staining with the 22E7 mAb compared with that obtained with IgG_1 control mAb. A clear population shift to the right is observed in both cases, indicating some specific binding of 22E7 by FcεRI on these cells (smf = 0.310 for monocytes, 0.120 for eosinophils).

RESULTS

The specific fluorescence emitted by basophils stained with both 22E7 and 15-1 was high and almost two logarithmic decades greater than that of the isotype control. However, particularly on eosinophils but occasionally also on monocytes, the specific staining on cells from some subjects was of a similar order to staining with the control antibody, although it was still possible to differentiate between specific and nonspecific staining in most cases (Fig. 1). Moreover, analyses of replicate eosinophil samples indicated that this staining was very reproducible: the coefficients of variation for IgG_1 and 22E7 mfis were only 0.67% and 0.40%, respectively. However, with an average mfi of 0.47 when cells were stained with the isotype control mAb in this study, even such small variability could result in some false-positive results when FcεRI expression was extremely reduced (i.e., at smf values of 0.01 or less). Consequently, only smf values exceeding 0.1 were considered to reliably represent FcεRIα–specific staining in this study.

The smf values of peripheral blood basophils, monocytes, and eosinophils from atopic and nonatopic subjects after staining with 22E7 are shown in Fig. 2. When all 38 subjects were considered together, the average cell-surface FcεRI expression (as determined by smf of 22E7 staining) was almost 500 times greater on basophils (median smf, 61.3) than on monocytes (median smf, 0.13). Monocytes in turn had higher FcεRI expression than eosinophils. In fact, in the majority of subjects, there was no 22E7-specific staining on eosinophils (median smf, <0.1). Nevertheless, there were a few individuals, almost exclusively atopic subjects, whose eosinophils clearly had specific staining (although this was of a greatly reduced magnitude compared with staining on basophils). The histograms shown in Fig. 1, *B*, are from one such individual. The smf of 22E7 staining was significantly higher in atopic subjects than in normal subjects on each cell type: the median smf values for atopic subjects versus nonatopic subjects were 75.6 versus 29.6 ($p < 0.0001$) for basophils, 0.45 versus <0.1 ($p = 0.003$) for monocytes, and 0.15 versus <0.1 ($p = 0.039$) for eosinophils.

With 15-1, the intensity of specific staining was markedly lower on all three cell types, and monocytes and eosinophils from a greater proportion of both atopic and nonatopic subjects failed to show any specific staining (the median smf values were 4.3 on basophils and <0.1

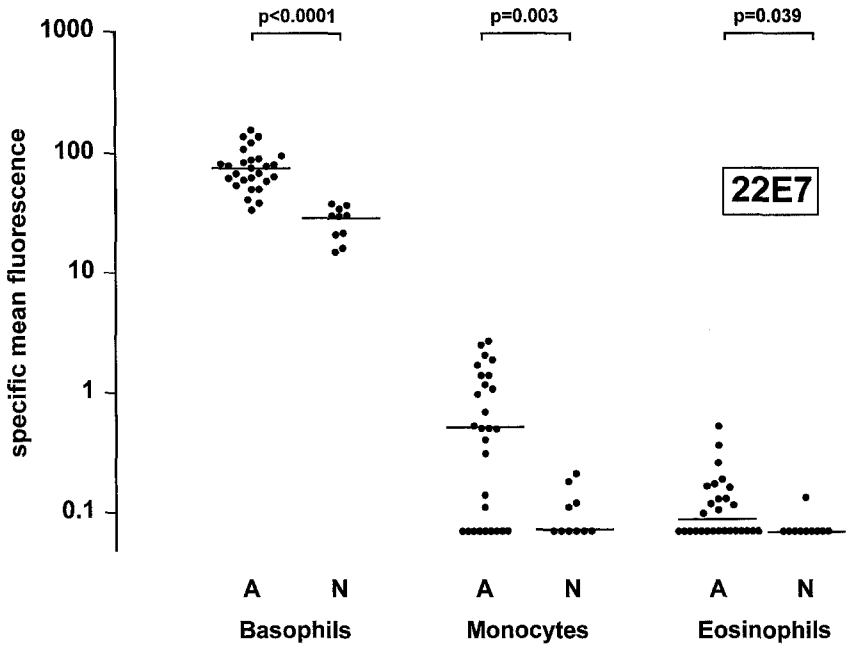

FIG. 2. Comparison of smf values of 22E7 binding (plotted on a logarithmic scale) by peripheral blood basophils, monocytes, and eosinophils from atopic *(A)* and normal *(N)* donors. Bars show median smf values.

on monocytes and eosinophils). There were no significant differences between atopic subjects and normal subjects with regard to 15-1 smf on monocytes and eosinophils but significantly lower 15-1 smf values on basophils from atopic subjects ($p = 0.0002$) (Fig. 3). For every subject, whether atopic or normal, 22E7 smf was greater than or (when there was no specific binding) equal to 15-1 smf on all three cell types.

The median and ranges of the smf values for both 22E7 and 15-1 staining on basophils and monocytes from the three groups of patients with primary diagnoses of AA, AR, or AD and the normal, nonatopic control subjects are shown in Table II. Significant variability in the smf of basophils after 22E7 staining was observed ($p = 0.04$ among the four groups by Kruskal-Wallis ANOVAR). Significantly lower smf of basophils after staining with 22E7 was found in the normal control group compared with each group of atopic subjects ($p \leq 0.0004$). There was considerable overlap between 22E7 smf on basophils from the three groups of atopic patients, although the median smf was highest in the patients with AD. Basophil smf after 15-1 staining also showed significant variability ($p = 0.04$ by Kruskal-Wallis) and a broadly reciprocal pattern of staining between each group to that seen with 22E7, with the highest median 15-1 smf observed in the normal control subjects.

Similarly, there was significant variability of smf ($p = 0.001$ by Kruskal-Wallis) after 22E7 staining of monocytes from the four groups. Once again, there was considerable overlap between specific staining with 22E7 among each of the atopic groups, with the highest median smf observed in patients from the AD group. The smf values were elevated on monocytes from the three groups of atopic patients compared with those from normal control subjects, although this difference was not statistically significant for the comparison with the AR group. In contrast, the 15-1 smf on monocytes from all four groups did not show significant variability ($p = 0.18$ by Kruskal-Wallis).

When basophil 22E7 smf values from all the subjects were plotted against the logarithmic values of their total serum IgE (to accommodate the large range of serum IgE), there was significant positive correlation between log [serum IgE] and 22E7 smf ($r = 0.86$, $p < 0.001$). Similarly, a significant positive correlation was observed between log [serum IgE] and 22E7 smf on monocytes ($r = 0.55$, $p < 0.001$) (Fig. 4). The degree of binding of both anti-FcεRI mAbs to eosinophils was too low to allow correlations with serum IgE concentrations.

DISCUSSION

This study, with two fully characterized mAbs specific for the α-chain of the FcεRI, confirmed the presence of high-affinity IgE receptors on peripheral blood monocytes and eosinophils, as well as basophils, from both normal and atopic subjects. Receptor expression appeared to be substantially greater on basophils than on monocytes; and FcεRI expression on circulating eosinophils, if detected at all, was extremely reduced compared with that of both basophils and monocytes. Furthermore, the atopic subjects had significantly elevated FcεRIα expression, as assessed by the smf of 22E7

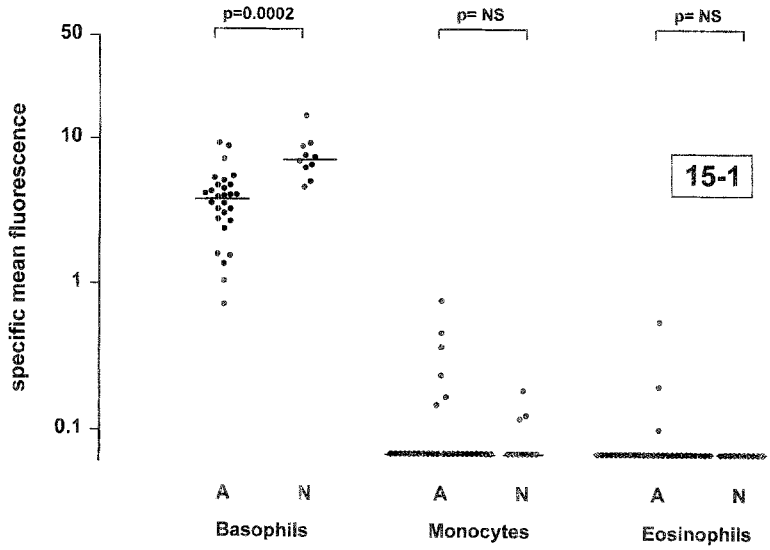

FIG. 3. Comparison of smf values of 15-1 binding (plotted on a logarithmic scale) by peripheral blood basophils and monocytes from atopic *(A)* and normal *(N)* donors. Bars show median smf values.

staining, on all three cell types compared with nonatopic control subjects.

Investigators studying FcεRI expression on blood monocytes from atopic subjects have previously used the 15-1 mAb.[7, 17] In this study the use of a combination of 22E7, a noncompetitive anti-FcεRIα antibody, as well as 15-1, which competes with IgE for binding FcεRIα and therefore binds only to unoccupied receptors, allowed assessment of FcεRI expression on cells stained immediately after isolation from whole blood without the requirement for dissociation of receptor-bound IgE. Comparisons of 22E7 and 15-1 smf values in these circumstances gave some indication of receptor occupancy in vivo. Not only was 22E7 smf almost invariably greater than 15-1 smf on all three cell types, but it was quite striking that, in contrast to 22E7, 15-1 specific staining on monocytes and basophils was not increased in atopic subjects compared with normal control subjects. In fact, on basophils, 15-1 smf in atopic subjects was actually slightly reduced. These observations suggest that the α-chains of FcεRI on these cells are to some extent occupied by IgE, and at least on monocytes and basophils, there was elevated occupancy of these receptors in atopic subjects. The validity of the assumption that the difference between 22E7 and 15-1 specific staining is a measure of receptor occupancy was tested in a separate experiment in which receptor-bound IgE on basophils and monocytes was dissociated by lactic acid (a modification of the method of Pruzansky et al.[18]). It was found that after dissociation, 15-1 smf values had increased and were now almost equivalent to 22E7 smf values (unpublished observations).

Interestingly, on both monocytes and basophils the degree of receptor expression in different individuals correlated highly significantly with serum IgE concentrations. Strikingly similar observations on basophil receptor expression have been made by Malveaux et al.[12] They quantified IgE receptors (the affinities of which were consistent with them being FcεRI) indirectly on basophils by first saturating leukocytes containing a known number of basophils with IgE and then dissociating the IgE and measuring the amount of eluted IgE. They found a highly significant correlation between total serum IgE concentrations and receptor expression on basophils from atopic and nonatopic donors. In addition, they also observed increasing occupancy with apparent saturation of receptors at serum IgE levels greater than about 400 ng/ml.[19] The suggestion that there is increased receptor occupancy on monocytes from atopic subjects is consistent with previous observations of the ability of FcεRI on monocytes from atopic subjects to bind IgE in vitro.[17, 20] A correlation between serum IgE concentrations and receptor expression on peripheral blood monocytes has not been previously described, although Maurer et al.[7] did find higher FcεRI expression on monocytes from subjects with AD or AR compared with nonatopic control subjects. The observation that despite the relative paucity of FcεRI expressed on circulating eosinophils, expression was nevertheless elevated on eosinophils from atopic subjects compared with those from nonatopic subjects is also novel.

Although the exact functional significance of elevated FcεRI expression on these cells in allergy is still unclear, evidence suggesting that these receptors could enhance their roles as effector cells in allergic inflammation is accumulating. Cross-linking of FcεRI on monocytes by mAbs specific for the α-chain, similar to those used in this study, has been observed to cause mediator release[20] and also to result in signal transduction,[7, 8] suggesting

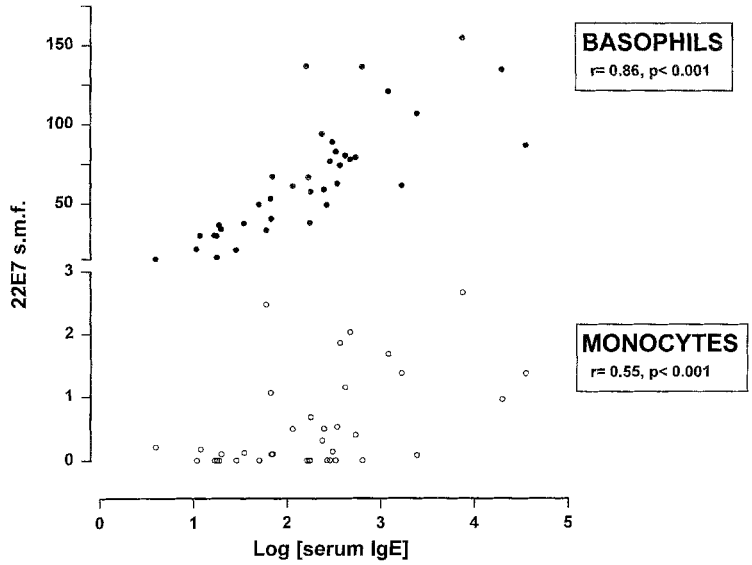

FIG. 4. Specific mean fluorescences of 22E7 binding by basophils and monocytes from all subjects plotted against logarithm of their total serum IgE concentrations.

TABLE II. The medians and ranges of smf values of the binding of mAbs 22E7 and 15-1 by basophils and monocytes obtained from nonatopic control subjects and patients with AA, AR, or AD

	NC		AA		AR		AD	
	Median	Range	Median	Range	Median	Range	Median	Range
Basophils								
22E7	29.6	14.8-37.4	64.9	37.8-93.8	63.8	33.2-136.9	96.8	61.6-154.8
15-1	7.2	4.6-14.4	4.0	2.7-9.4	4.1	2.4-5.1	1.6	0.7-8.9
Monocytes								
22E7	<0.1	<0.1-0.2	0.35	<0.1-1.2	<0.1	<0.1-2.5	1.2	<0.1-2.7
15-1	<0.1	<0.1-0.21	<0.1	<0.1-0.8	<0.1	<0.1-0.8	0.16	<0.1-0.7

NC, Normal control.

that the interaction of allergens with monomeric IgE bound to these receptors could have a role in transcriptional activation of proinflammatory cytokines such as IL-1 and tumor necrosis factor-α. It has also been observed that FcεRI binding of allergen-specific IgE on monocytes in vitro enables them to present antigen to T lymphocytes up to 1000-fold more effectively,[17] suggesting that elevated expression of FcεRI in atopic subjects might profoundly alter the spectrum of allergen-presenting cells available to present allergens to T cells. Cross-linking of receptor-bound IgE on basophils by allergen or anti-IgE also results in release of preformed mediators such as histamine. Increased basophil histamine releasability has been observed in atopic patients.[21] Moreover, basophils are also important sources of the cytokine IL-4, the synthesis and secretion of which is also IgE-dependent.[22]

Although the FcεRI-mediated release of proinflammatory mediator cytokines from eosinophils has not yet been demonstrated in atopy, in vitro functional activity of these receptors on eosinophils from patients with hypereosinophilia was demonstrated by FcεRIα involvement in eosinophil peroxidase release and IgE-dependent schistosomal killing.[10] Our observations suggest that in the majority of our patients with atopy, FcεRI expression on peripheral blood eosinophils was on the whole less than that previously described in patients with hypereosinophilic syndromes.[9] However, increased FcεRI expression has been described on tissue eosinophils at sites of allergic inflammation.[10, 11] These apparently contrasting observations might be explained by allergen stimulation causing either selective recruitment of FcεRI$^+$ eosinophils or, more likely, upregulation of these receptors on recruited eosinophils after interactions with cytokines such as IL-5, IL-3, or granulocyte-macrophage colony-stimulating factor and/or extracellular matrix with resultant cellular activation.[23] Evidence supporting either (or both) of these possibilities has been provided by a study, which showed that in the cutaneous late-phase reaction resulting from allergen challenge, there was recruitment of eosinophils, about 26% of which expressed FcεRI, whereas FcεRI$^+$ eosin-

ophils were not a feature of skin biopsy specimens from sites of diluent challenge.[24]

The correlation observed between serum IgE concentrations and high-affinity IgE receptor expression, at least on basophils and monocytes, is intriguing. One interpretation of these findings is that IgE itself upregulates or stabilizes surface expression of the receptor leading to elevated expression in atopy. It is also possible that elevated FcεRI expression is controlled by regulatory mechanisms similar to those that control IgE synthesis. Results of several recent studies have suggested a genetic linkage of atopic IgE responses to the gene for the β-chain of the high-affinity receptor for IgE on chromosome 11q13.[25-27] The observations made in this study might suggest an association at the phenotypic level as well.

In conclusion, we have observed significantly elevated FcεRI on circulating basophils, monocytes, and eosinophils in atopic subjects. At least on basophils and monocytes, FcεRI expression correlated significantly with serum IgE concentrations, raising the possibility of common control mechanisms. There also appeared to be increased occupancy in atopic subjects, suggesting a possible role for this receptor in enhancing the effector functions of these cells during IgE-dependent allergic inflammation.

We thank Dr. Richard Staughton of the Department of Dermatology, The Chelsea and Westminster Hospital, for his help in recruiting patients with atopic dermatitis. We also thank Drs. Jean-Pierre Kinet, Richard Chizzonite, and Jarema Kochan for kindly providing the anti-FcεRI antibodies and Drs. Douglas Robinson and Chris Corrigan for their critical appraisal of the manuscript.

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2.2 Addendum – Further Data On IgE Occupancy Of FcεRI On Basophils And Moncytes

In the discussion of the paper presented in Section 2.1, it was mentioned that the use of a non-competitive (22E7) and a competitive (15-1) anti-FcεRIα mAb at saturating concentrations in our study provided a good estimate of the level of occupancy of FcεRI by monovalent IgE. It was also stated that by assessing this estimated occupancy it became apparent that a greater proportion of FcεRIα chains were occupied on basophils and monocytes in atopic subjects than in non-atopic controls. However there were a number of assumptions involved in estimating occupancy by this method and therefore the method or data were not discussed further in the paper. Nevertheless it might be appropriate to describe these briefly at this stage since the importance, in these cell types, of the observation of a close inter-relationship between serum IgE and cell surface expression of the α-chain of its high affinity receptor will be discussed in some detail in Chapter 6.

The concept that the difference between the specific mean fluorescence intensity (smf) of staining of receptors by these two mAbs might provide a measure of receptor occupancy arose from the initially puzzling observation that in contrast to 22E7, whose smf levels on both cell types increased in atopic individuals with elevated serum IgE concentrations, 15-1 specific staining tended, if anything, to be slightly higher in non-atopic subjects and was at times barely detectable in subjects with higher serum IgE concentrations (as described in the paper). The most logical explanation was that even as there must be greater numbers of FcεRI expressed on these cells as IgE levels increase, more of these receptors must be bound to IgE – thus resulting in reduced 15-1 staining.

As saturating concentrations of mAbs were used, the non-competitive mAb 22E7 could be assumed to bind to all FcεRIα chains whether occupied or unoccupied by IgE, whereas 15-1, the competitive mAb, would bind only to unoccupied receptors. The specific mean fluorescence values of binding to 22E7 and 15-1 for each subject observed in the study therefore could be assumed to represent, respectively, total and unoccupied receptor expression. By implication therefore, as the cytometric

evaluation was done using standardised conditions and each mAb had the same number of fluorochromes of similar brightness, receptor occupancy should be directly proportional to the difference between the smf of receptor staining on cells from the same individual with the two mAbs and therefore it could be estimated quite accurately by expressing the difference between the smf of 22E7 and 15-1 staining of basophils as a percentage of 22E7 smf. The validity of these assumptions was tested in a separate pilot experiment described below.

The aim of this pilot (or 'proof of concept') study was to investigate the hypothesis that 15-1 smf on both basophils and monocytes would increase and become comparable to 22E7 smf when receptor bound IgE was dissociated from the receptors. Peripheral blood mononuclear cells (PBMC) from 8 subjects (6 atopic and 2 non-atopic) were studied and IgE was dissociated from FcεRI by a modification of the acid elution technique first described by Pruzansky (Pruzansky et al. 1983). This method efficiently removes most (at least 95%) of the receptor bound IgE from the surface of living cells whilst maintaining cell structure and integrity.

The method, briefly, was that PBMC obtained from whole blood by the Dextran-Ficoll gradient method (described in Section 2.1 – 'Study Design') were washed twice in PAG buffer (an isotonic organic buffer consisting of 25 mM PIPES buffer/0.1% human albumin solution/0.1% glucose), resuspended in PBS (phosphate buffered saline) and a sample taken for a cell count. Around 4 x 10^6 PBMC were then removed and left at 4^{o}C until the time came for staining with the FcεRI specific mAbs for analysis (these were the untreated cells from which IgE had not been dissociated). The remaining PBMC were then recovered from solution by centrifugation and were then subjected to the elution procedure by being resuspended in 5 ml 0.01M lactic acid (pH 3.9) at room temperature. After 3.5 minutes the elution process was immediately stopped by the addition of 35 ml PAG buffer. Both the untreated and treated PBMC samples were now recovered from solution by centrifugation and washed twice in PAB (PBS containing 0.5% wt/vol bovine serum albumin and 0.1% wt/vol sodium azide) before being resuspended in PAB at a concentration of 1 x 10^6 PBMC per 100 μl. The cells then underwent the same triple stage staining procedure with mAbs that

is described in Section 2.1 – 'Study Design' and were then analysed by flow cytometry – again exactly as described in Section 2.1 – 'Flow cytometry'.

Most interestingly, the results of this study (*shown in Figures 1 and 2 below*) demonstrated that although, as expected, 15-1 smf was significantly less than 22E7 smf on the untreated cells (both basophils and monocytes), after the removal of receptor bound IgE (by elution with 0.01M lactic acid) the smf of 15-1 staining indeed increased and became almost equivalent to that of 22E7. Moreover there was no significant difference between 22E7 smf on these cells pre- or post-dissociation, implying that there was no significant receptor loss.

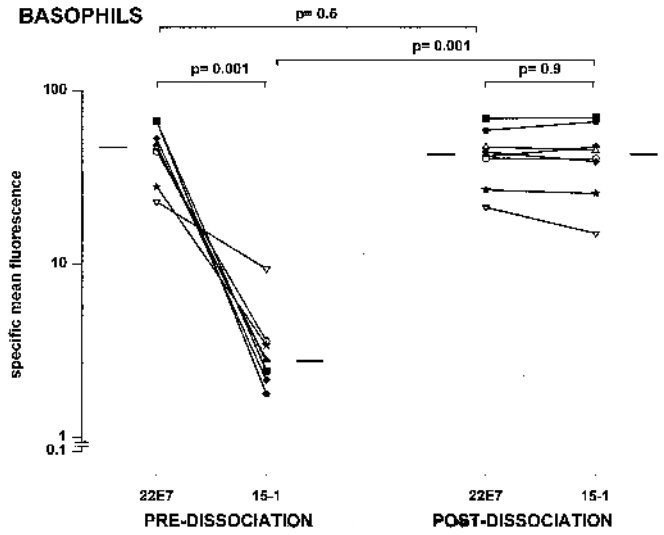

Figure 1: Specific mean fluorescence values of basophil staining with 22E7 and 15-1 pre- and post- dissociation of receptor-bound IgE by 0.01M lactic acid

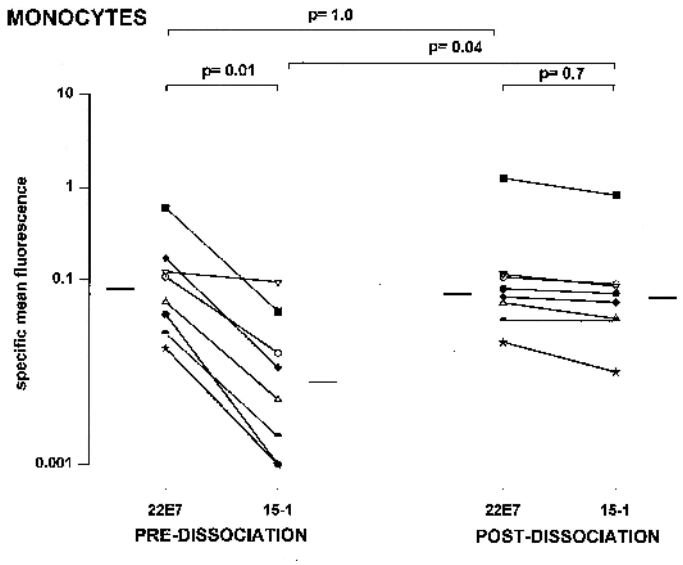

Figure 2: Specific mean fluorescence values of monocyte staining with 22E7 and 15-1 pre- and post-dissociation of receptor-bound IgE by 0.01M lactic acid

This study did therefore provide evidence to support the concept that the difference between the smf values for 22E7 and 15-1, expressed as a percent of 22E7 smf, would indeed provide a very good approximation of receptor occupancy on both basophils and monocytes in the subjects participating in the study described in Section 2.1.

The percent FcεRI occupancy on basophils (calculated as above) for each subject was then plotted against that subject's serum IgE concentration (*figure 3*). Even in non-atopic subjects, with low serum IgE concentrations, the majority of receptors (about 60%) were occupied and receptor occupancy increased as serum IgE concentrations increased until receptor saturation was observed at serum concentrations of greater than about 400 IU/ml.

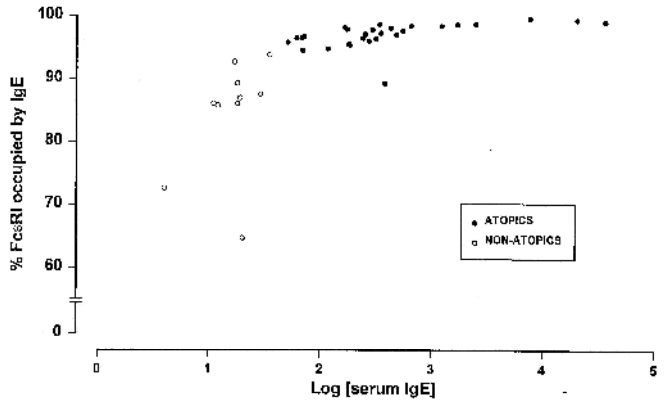

Figure 3: Percent occupancy of high affinity Fc receptors by IgE on basophils from atopic and normal donors plotted against the logarithm of their total serum IgE concentrations

Furthermore, using this same method to calculate receptor occupancy on monocytes, we observed once again that occupancy appeared to be related to serum IgE concentrations – albeit that this relationship was not quite as clearly obvious as for basophils (*figure 4*) – with receptor saturation appearing to occur at IgE levels of around 300-400 IU/ml and occupancy at lower serum IgE concentrations apparently much reduced in comparison to basophils.

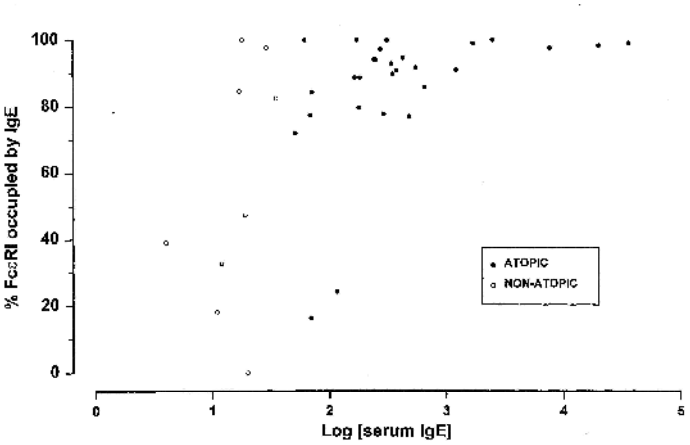

Figure 4: Percent occupancy of high affinity Fc receptors by IgE on monocytes from atopic and normal donors plotted against the logarithm of their total serum IgE concentrations

Whilst this method of assessing receptor occupancy was of course somewhat convoluted and indirect, the findings described above suggested that the main observation of the study (that FcεRI expression on basophils and, to a lesser but still significant extent, monocytes correlates closely with serum IgE concentrations) was more than mere coincidence. Indeed they suggested the strong possibility of a well-defined causal relationship between IgE binding and FcεRI expression on these cells – a possibility that is considered further in Chapter 6.

Chapter 3

Basophils and Interleukin-4

3.1 Unstimulated Basophils In Atopic And Non-Atopic Subjects Express Intracellular Interleukin-4: Detection By Flow Cytometry

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Short communication

Unstimulated basophils in atopic and nonatopic subjects express intracellular interleukin-4: detection by flow cytometry

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Background IgE-stimulated cultured basophils from atopic subjects are capable of secreting interleukin-4 (IL-4). We describe a flow-cytometric technique which identified intracellular IL-4 in unstimulated basophils unseparated from peripheral blood mononuclear cells (PBMC) in both atopic (AT) and nonatopic (NC) volunteers.
Methods Freshly isolated PBMC were fixed in 4% paraformaldehyde (PFA). Surface staining with 22E7, a noncompetitive anti-$Fc_{\varepsilon}RI$-α antibody, allowed identification of basophils. Permeabilization by 0.1% saponin allowed staining of intracellular cytokines with specific monoclonal antibodies (mAbs). Two series of experiments utilizing different protocols and anticytokine mAbs were performed. The first protocol required a two-stage fluorochrome staining technique. The availability of fluorochrome-conjugated mAbs allowed a simpler, one-stage labelling procedure for the second protocol.
Results With the first protocol, IL-4 (but not IFN-γ), immunoreactivity was detectable in a majority (median 77%) of peripheral blood basophils from both AT and NC subjects (n=8). Basophil IL-4 immunoreactivity was again evident in experiment 2 but did not differ significantly between AT and NC subjects – either evaluated as percentage of IL-4+ basophils (AT median=66%, NC median=38.4%, P=0.41) or IL-4-specific mean fluorescence (AT median=0.85, NC median=0.3, P=0.07).
Conclusions This simple technique allowed the study of intracellular cytokine expression in unstimulated blood basophils. It demonstrated constitutive basophil expression of IL-4 (but not IFN-γ) in all subjects, with no significant increases in atopics.

Key words: basophils; cytokines; flow cytometry; interleukin-4.

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Several recent studies have demonstrated that partially purified peripheral blood basophils may generate and release interleukin-4 (IL-4) after IgE-dependent (1–4) and IgE-independent activation (5). There is also some evidence that unstimulated basophils may contain preformed IL-4 (3, 6). The possibility that basophils are sources of rapidly mobilizable T_H2-type cytokines extends their potential role in allergic inflammation (7). However, the relationship of atopic status to the production of IL-4 by unstimulated basophils is still unclear.

In this study, we used a rapid flow-cytometric method, which does not require purification or stimulation of peripheral blood basophils, to assess intracellular expression of IL-4 and interferon-gamma (IFN-γ) in atopic and nonatopic volunteers. This was a combination of previously described methods for the flow-cytometric detection of intracellular cytokines (8) and differentiation of basophils from other PBMC (9). This technique makes use of the uniquely high level of basophil binding of 22E7, a noncompetitive murine monoclonal antibody (mAb) specific for

the α-chain of the high-affinity Fc receptor for IgE ($Fc_\varepsilon RI$).

Material and methods

Subjects

Volunteers were recruited from the allergy clinic at the Royal Brompton Hospital (London, UK). Atopy was defined as either a positive skin prick test (wheal diameter of >3 mm compared to diluent control) or a positive RAST test (>0.70 IU/ml) to one or more of a panel of common aeroallergens (including house-dust mite, mixed grass pollen, mixed tree pollen, mixed moulds, and cat and dog fur). In addition, all the atopic subjects (AT) gave a clear history consistent with allergic asthma or rhinitis. None of the subjects were taking any regular inhaled or intranasal anti-inflammatory medication, and any who had received oral steroids within the preceding 3 months or immunotherapy during the preceding 12 months were excluded from the study. Nonatopic control subjects (NC) had no clinical or laboratory evidence of atopy.

Two series of experiments were performed (studies 1 and 2), each using different subjects and different cytokine-specific mAbs. Study 1 involved only eight subjects (five AT and three NC), and its objective was to confirm the feasibility of using this technique to detect intracellular cytokines in blood basophils. A two-stage staining method was used to label indirectly the murine primary mAbs with fluorochrome-conjugated goat antimurine antibodies.

The subsequent study (study 2) involved a more detailed comparison of basophil cytokine expression between atopic subjects (AT) and nonatopic controls (NC). This used a simpler one-stage staining method, as directly conjugated mAbs were now available.

Ten AT (five atopic rhinitics and five atopic asthmatics) with a median age of 32.5 years (range 20–57) and median total serum IgE of 269 IU/ml (range 41–831) were studied. The AT group comprised seven men and three women. Also studied were seven nonatopic controls (NC) (three men and 4 four women) with a median age of 29 years (range 25–32) and median total serum IgE of 29 IU/ml (range 11–467).

For both studies, peripheral blood mononuclear cells (PBMC) were isolated from 10 ml of freshly obtained heparinized peripheral venous blood by density gradient centrifugation over Histopaque (Sigma, UK) and immediately fixed by suspension for 10 min in 4% paraformaldehyde (BDH, UK) in phosphate-buffered saline (PBS) at 4±0.5°C.

Subsequent steps in both studies were all performed at ambient temperature (20±2°C). This included permeabilization of cell membranes with 0.1% saponin (Sigma, Poole, UK) solution in PBS containing 10% mixed human serum ("saponin buffer").

Monoclonal antibodies (mAbs)

All the primary mAbs used were murine isotype origin, isotype IgG1. The 22E7 was a kind gift from Drs R. Chizzonite and J. Kochan, Hoffman-La Roche Inc., Nutley, NJ, USA. Although indirect immunofluorescence was used to detect this antibody in study 1, for study 2 it was directly conjugated to fluorescein isothiocyanate (FITC). The conjugation was performed by Cymbus Biotechnology Ltd, Southampton, UK.

Study 1. The IL-4-specific mAb, 4D9, was a kind gift from Dr C. Heusser, Ciba Geigy, Basel, Switzerland, and the IFN-γ-specific clone, GZ-4, was obtained from Boehringer Mannheim, Germany. An isotype-identical irrelevant control mAb was obtained from Dako, Glostrup, Denmark. Counterstaining of 22E7 was done with a second-layer FITC-conjugated goat antimurine Ig $F(ab)_2$ antibody, and of the anticytokine mAbs with a phycoerythrin (PE)-conjugated goat antimurine IgG1 antibody. Both secondary mAbs were obtained from Caltag Laboratories, San Francisco, CA, USA.

Study 2. The anticytokine mAbs for the second study were all directly conjugated to PE by PharMingen, San Diego, CA, USA. They were clone 8D4-8 (IL-4-specific), clone 4S.B3 (IFN-γ-specific), and an irrelevant isotype control (clone MOPC-21).

Staining protocols

Study 1. Aliquots of 10^6 PBMC permeabilized in saponin buffer were incubated for 40 min with the unconjugated cytokine-specific mAb (4D9 or GZ-4) or the isotype control (at a final antibody concentration of 10 μg/ml), and then washed twice in saponin buffer and incubated in the dark for a further 40 min with the PE-conjugated secondary mAb (diluted 1:40 in saponin buffer). The cells were then washed twice in saponin buffer and twice in PAB (PBS containing 0.5% w/v bovine serum albumin and 0.1% sodium azide) to reverse the permeabilization. Cells were suspended in PAB and then surface stained by sequential 20-min incubations in the dark with 22E7 (at a final concentration of 10 μg/ml) and then with the FITC-conjugated secondary antibody (diluted 1:40 in PAB). Surface staining was deferred until permeabilization had been reversed so as to prevent

staining of 22E7 (a murine mAb of identical isotype to the cytokine-specific mAbs) by the PE-conjugated secondary antibody used to stain the anticytokine mAbs. After a final wash, the cells were resuspended in 250 µl 0.5% formaldehyde solution in Isoton (Coulter, USA), ready for analysis by flow cytometry.

The necessity for a two-stage staining procedure in study 1, with the ensuing requirements for intermediate "blocking" steps to prevent nonspecific staining, resulted in a complex sequence of staining and permeabilization steps. We therefore evaluated a more rapid and less complex staining method with the availibility of directly conjugated mAbs in study 2.

Study 2. The final concentrations of all mAbs used in this protocol was 10 µg/ml. The risk of cross-reactivity was minimal, as directly conjugated mAbs were used. In preliminary experiments assessing staining with 22E7-FITC before or after permeabilization, the best definition was achieved with staining before permeabilization (with no effect on intracellular staining intensity). Therefore, in this protocol, aliquots of 10^6 PBMC were first surface stained by incubation with 22E7-FITC in the dark for 30 min and then washing twice with PAB before permeabilization with saponin buffer as before. Permeabilized cells in saponin buffer were then incubated in the dark for 30 min with the PE-conjugated IL-4-specific or isotype control mAbs. Cells from four of these subjects were also incubated similarly with the PE-conjugated anti-IFN-γ mAb. After two further washes in PAB, the cells were resuspended in 250 µl 0.5% formaldehyde solution in Isoton.

Flow cytometry

The samples were analysed on a Coulter Epics Elite cell sorter and flow cytometer (Coulter Corporation, Hialeah, FL, USA) fitted with a 488 nm argon laser and equipped with 520 nm (FITC) and 575 nm (PE) bandpass filters. Identical fluorescence gain and compensation settings were used for each series of experiments, and the cytometer flow cell was fully aligned and calibrated with DNA-Check fluorospheres (Coulter) before each run.

The technique of using flow cytometry to identify basophils in PBMC samples by their characteristic side-scatter (SSC) and very high surface expression of $Fc_\varepsilon RI$-α has been detailed previously (9). Briefly, a plot of $Fc_\varepsilon RI$-α expression (assessed by 22E7 binding) against SSC usually revealed four distinct cell populations – lymphocytes, monocytes, basophils, and blood dendritic cells (Fig. 1a). The identities of these cell populations were confirmed by sorting and also by immunophenotyping. Thus, T and B cells are $Fc_\varepsilon RI^{absent}$ but $CD2^{high}$, and $CD20^{high}$, respectively; monocytes are $Fc_\varepsilon RI^{moderate}$, $CD14^{high}$, and HLA-DR^{high}; blood dendritic cells are $Fc_\varepsilon RI^{high}$, $CD14^{low}$, and HLA-$DR^{very\ high}$ (confirmed by Maurer et al. [10]); and basophils are $Fc_\varepsilon RI^{very\ high}$, $CD14^{absent}$, and HLA-DR^{absent}. Therefore, intracellular cytokine expression in unseparated basophils could be analysed by gating on the appropriate cell population.

Data were collected from 2000 basophils per sample. The percentages of IL-4-immunoreactive basophils were analysed by the Immuno-4 software program (Coulter). This program used the Overton method of channel-by-channel histogram subtraction (11) to compare histograms generated by cells

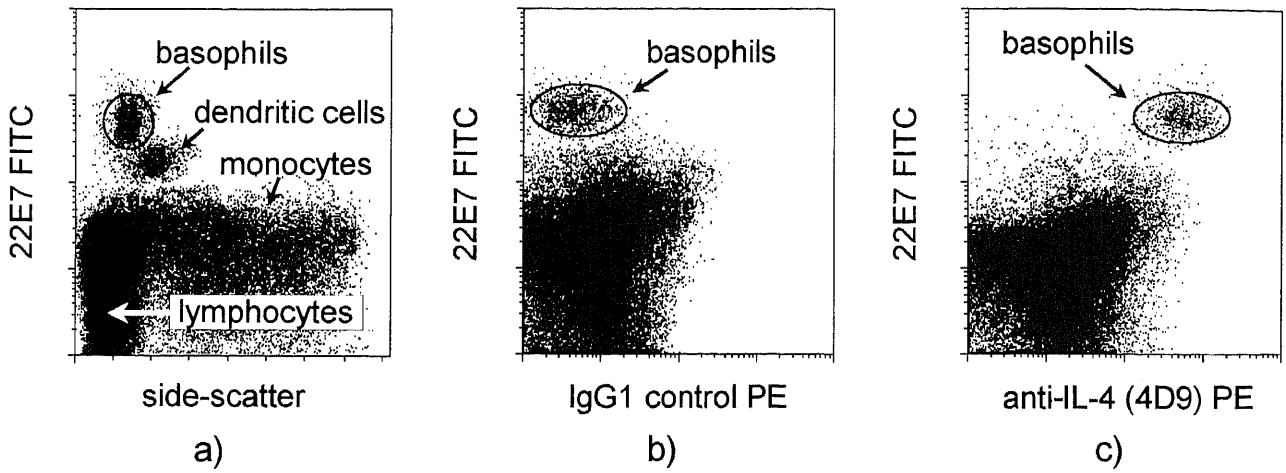

Fig. 1. Characteristic dual parameter histograms of single subject, showing a) surface $Fc_\varepsilon RI$-α expression (by 22E7 FITC) against side-scatter (SSC) showing four distinct cell populations: basophils, dendritic cells, monocytes, and lymphocytes; b) negative and c) positive intracellular staining with isotype control and anti-IL-4 (4D9) antibodies, respectively, only in basophils.

stained with the anti-IL-4 mAb and an identical number of cells stained with the isotype control. In addition, the specific mean fluorescence (s.m.f.) – the difference between the mean fluorescence intensity of cells stained with the cytokine-specific mAb and the mean fluorescence intensity of these cells stained with the isotype-identical irrelevant control antibody – was determined as a measure of the intensity of cytokine-specific staining.

Statistical analysis

Data were analysed by a statistical software package (Minitab for Windows, Release 9.2, Minitab Inc., State College, PA, USA). The Mann–Whitney U-test, a nonparametric analytical test, was used to analyse intergroup differences. For all comparisons, P values less than 0.05 were considered significant.

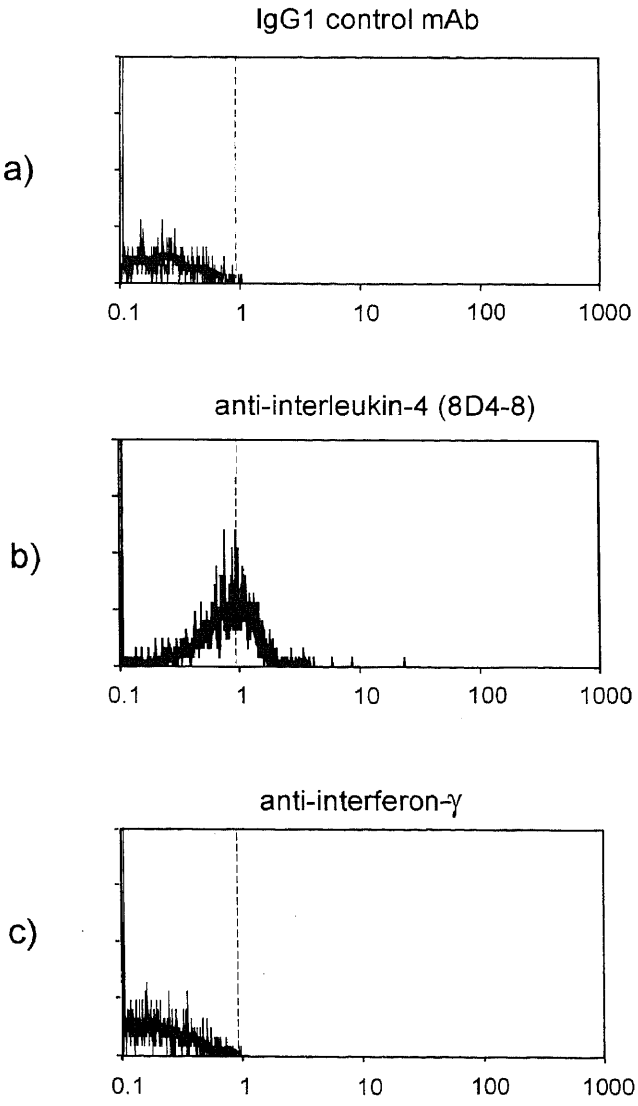

Fig. 2. Representative histograms demonstrating basophil staining with a) control, b) anti-IL-4, and c) anti-IFN-γ mAbs, and showing IL-4, but not IFN-γ, specific staining.

Results

Study 1

Compared to the isotype control mAb, basophil IL-4 (4D9), but not IFN-γ, immunoreactivity was clearly demonstrable in every subject in this study (Fig. 1). The individual values for the percentages of basophils staining positively with this IL-4-specific mAb compared to the isotype-identical control mAb were 55%, 68%, 76%, 76%, 77%, 88%, 91%, and 91% (median 77%). IL-4 immunoreactivity was not demonstrable in any of the other cell types in these PBMC samples.

Study 2

IL-4 immunoreactivity in unstimulated basophils was also demonstrable with 8D4-8 (Fig. 2). Once again, a majority of basophils in both subject groups appeared to have intracellular, immunoreactive IL-4. The percentages of basophils staining positively with 8D4-8 in the AT group ranged from 21.5% to 84.3% (median 66.0%) and in the NC group from 6.9% to 77.8% (median 38.4%) (Fig. 3a). The percentages of basophils showing IL-4 immunoreactivity did not differ significantly between these two subject groups (P=0.41). The intensity of the IL-4-specific staining tended to be greater in the AT group, but the intergroup difference just failed to achieve statistical significance (P=0.07). The median s.m.f. value was 0.85 (range 0.14–2.42) for the atopic group and 0.3 (range 0.02–0.87) for NC subjects (Fig. 3b). Once again, no basophil IFN-γ immunoreactivity was observed in this study (Fig. 2).

Discussion

The flow-cytometric technique we describe is both rapid and sensitive and may be used to identify unseparated peripheral blood basophils at high purity, thus allowing the detection of intracellular cytokine protein in intact unstimulated cells. Therefore, it allows the investigation of the expression of such proteins in *in vivo* situations, as there is no requirement for stimulation of these cells before analysis. This method by definition assesses only those basophils showing elevated expression of $Fc_{\varepsilon}RI$. It could be argued that a subset of basophils with relatively low $Fc_{\varepsilon}RI$ expression and potentially different cytokine expression characteristics might therefore escape analysis. In our view, this is unlikely, since we and other groups (9, 10) have

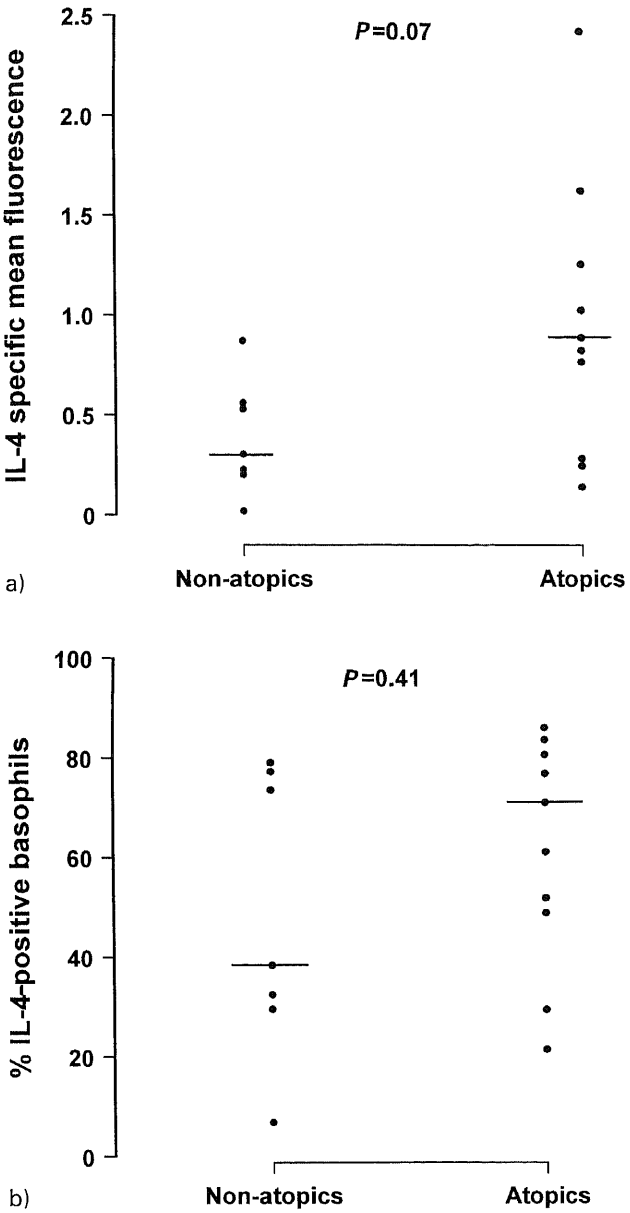

Fig. 3a. Specific mean fluorescence of IL-4 in basophils in atopic subjects and nonatopic controls. *Fig. 3b.* Percentage of basophils that express IL-4 in atopic subjects and nonatopic controls.

assessed the groups of cells with relatively lower $Fc_{\varepsilon}RI$ expression and have shown these to be dendritic cells with distinct flow-cytometric characteristics and with no evidence of a separate low-expression basophil group.

We have observed constitutive IL-4 protein expression in unstimulated basophils from atopic and nonatopic subjects with two experimental protocols and different mAbs. These findings are consistent with a recent study which demonstrated the rapid release of preformed IL-4 from anti-IgE-stimulated basophils (6). Using confocal scanning fluorescent microscopy, Mueller et al. also observed IL-4 immunoreactivity in 6–9% of unstimulated basophils from nonatopic donors (3). Furthermore, they observed detectable amounts of IL-4 in the supernatants of unstimulated cells. This is in contrast to previous studies suggesting that basophils constitutively express IL-4 mRNA but do not store the preformed protein although they were capable of secreting newly synthesized IL-4 after IgE stimulation (2, 4).

In our study, the percentages of basophils that expressed IL-4 protein constitutively were relatively increased as compared to those reported by Mueller et al. (3), a fact which may reflect the different sensitivities of the techniques used as well as the larger numbers of cells examined. Furthermore, we assessed cells in freshly venesected peripheral blood, whereas, in the previous study, purified cells were incubated for 18 h before analysis, with measurable amounts of IL-4 loss into the supernatant. The use of directly conjugated mAbs may be less sensitive, because of the lack of an amplification step, as compared to the initial protocol which employed a two-stage staining protocol, and may explain the higher staining values observed with our initial experimental methodology in this study.

There was no significant increase in the intensity of IL-4 immunoreactivity (as assessed by specific mean fluorescence) or in the overall percentages of IL-4-positive cells when unstimulated basophils were compared in atopic and nonatopic subjects, although there appeared to be a trend to increased specific mean fluorescence in atopic subjects. This study did not address the effect of anti-IgE or cross-linking of $Fc_{\varepsilon}RI$, but it is notable that Schroeder et al. (4) were unable to demonstrate any significant differences in IgE-dependent basophil IL-4 secretion in atopic and nonatopic subjects.

In conclusion, this relatively rapid and simple method for detecting intracellular cytokine products in unseparated and unstimulated basophils was used to demonstrate IL-4-specific immunoreactivity in both atopic and nonatopic subjects. Atopics have elevated levels of allergen-specific IgE, and their basophils possess elevated numbers of $Fc_{\varepsilon}RI$ (9), show increased IgE-dependent histamine releasability (12), and infiltrate sites of allergic inflammation (13). Thus, the presence of IL-4 stored intracellularly in unstimulated basophils suggests that they may potentially provide a rapidly releasable source of this cytokine through IgE-dependent activation. These findings are therefore consistent with the hypothesis that these cells may play a role in the initiation of allergic inflammation.

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Chapter 4

Cyclosporin A And Allergen-Induced Early And Late Phase Asthmatic Reactions

4.1 Effect Of Cyclosporin A On The Allergen-Induced Late Asthmatic Reaction

(published in Thorax 1997; 52: 447-452)

Effect of cyclosporin A on the allergen-induced late asthmatic reaction

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Abstract

***Background* – The allergen-induced late asthmatic reaction (LAR) is associated with mucosal inflammation involving several cell types including activated T lymphocytes and eosinophils. In contrast, the early asthmatic reaction (EAR) is considered to result from rapid allergen-induced release of bronchoconstrictor mediators from IgE sensitised mast cells. Cyclosporin A has efficacy in chronic severe corticosteroid-dependent asthma and is believed to act principally by inhibiting cytokine mRNA transcription in T lymphocytes. However, it has effects on other cell types in vitro, including the inhibition of exocytosis/degranulation events in mast cells. It was therefore hypothesised that cyclosporin A would attenuate both the EAR and LAR in subjects with mild asthma.**

***Methods* – Twelve sensitised atopic asthmatic subjects with documented dual asthmatic responses were studied in a double blind, placebo controlled, crossover trial. On two separate study visits subjects received two oral doses of either cyclosporin A or matched placebo before inhaled allergen challenges. The forced expiratory volume in one second (FEV_1) was measured half hourly for eight hours and blood eosinophil counts were analysed three, six, and 24 hours after the challenge. Treatment effects on blood eosinophil counts as well as the EAR and LAR, respectively defined as the areas under the curve (AUC) of FEV_1 changes from baseline between 0–1 and 4–8 hours after challenge, were compared by non-parametric crossover analysis.**

***Results* – Cyclosporin A reduced both the LAR (median AUC −41.9 l.h (interquartile range −82.7 to −12.4) for cyclosporin A and −84.5 l.h (−248.9 to −39.1) for placebo; $p=0.007$) and the late increase in blood eosinophils (median 0.2×10^9/l (0.15 to 0.4) for cyclosporin A and 0.4×10^9/l (0.25 to 0.55) for placebo; $p=0.024$) but had no effect on the EAR. The reduction of the LAR by cyclosporin A correlated significantly with prechallenge blood concentrations of cyclosporin A ($r=0.6$, $p=0.028$).**

***Conclusions* – These data are consistent with the concept that cyclosporin A has anti-inflammatory actions in asthma resulting from inhibition of mRNA transcription of eosinophil-active cytokines, predominantly in T lymphocytes. Cyclosporin A, possibly in its inhaled form, or other agents which prevent cytokine gene transcription may therefore have potential in ameliorating the inflammatory component of asthma.**

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It is well recognised that bronchial allergen challenge of appropriately sensitised atopic asthmatic subjects provokes an immediate bronchoconstrictor response (usually within 30 minutes) which resolves within 1–2 hours. This is known as the early asthmatic reaction (EAR). In some asthmatic subjects this is followed by a more sustained delayed-in-time late phase asthmatic reaction (LAR) which peaks within eight hours and resolves within 24 hours (although an associated increase in bronchial hyperresponsiveness may last for several days).[1]

The mechanisms of EAR and LAR to inhaled allergen and the associated increase in bronchial hyperresponsiveness have been extensively studied. The EAR is believed to result primarily from the rapid release of preformed histamine and newly generated lipid mediators such as cysteinyl leukotrienes and prostanoids[2,3] from IgE sensitised mast cells. In contrast, evidence from a number of studies suggests that the LAR may be attributable to mucosal infiltration with inflammatory cells.[4–6] Particular features of this bronchial inflammatory response include T lymphocyte activation, local eosinophil accumulation, and increased production of "eosinophil-active" cytokines.[5–7] This is associated with increased numbers of peripheral blood eosinophils.[8,9] The mechanism by which eosinophils are believed to contribute to the sustained LAR may be the result of the elaboration of cysteinyl leukotrienes which cause smooth muscle contraction, mucosal oedema, and mucus hypersecretion. In addition, release of basic proteins from secondary granules is believed to contribute to airway hyperresponsiveness (reviewed by Wardlaw *et al.*[10] Thus, the LAR is generally considered to be a model of mucosal inflammation now recognised as an integral part of the asthma process even in patients with mild disease.[11] The separate mechanisms suggested for the EAR and LAR are reflected in the different profiles of inhibition by pharmacological agents – for example, short acting β_2 agonists inhibit the EAR whereas glucocorticoids block the LAR.[12]

Table 1 Patient demographic data

Subject no.	Sex	Age (years)	Weight (kg)	Regular medication	Baseline FEV_1 (% predicted)	Challenge allergen	PC_{20} allergen (BU/ml)
1	F	45	55.9	B	108	C	14000
2	F	24	55.1	B, IS	92	HDM	1100
3	F	24	60.0	B, IS	95	C	12250
4	M	39	70.0	B	90	TGP	2500
5	F	28	52.9	B	113	C	8000
6	F	23	68.0	B, IS	110	HDM	12500
7	M	23	78.0	B, NS	107	HDM	2400
8	M	25	89.0	B	92	HDM	1000
9	F	26	59.7	B	105	TGP	2200
10	F	24	95.4	B	95	C	10400
11	M	35	69.3	B, IS	96	C	5000
12	M	42	111.0	B	102	TGP	1000

B = short acting β agonist; IS = inhaled corticosteroid; NS = intranasal corticosteroid; C = cat dander; HDM = house dust mite; TGP = timothy grass pollen; FEV_1 = forced expiratory volume in one second; PC_{20} = concentration of allergen provoking a fall of 20% in FEV_1.

Low doses of the immunosuppressive agent cyclosporin A have been found to be effective in the treatment of a number of chronic inflammatory diseases characterised by T lymphocyte activation[13–16] and there is accumulating evidence for a role for activated T cells in the pathogenesis of asthma.[17–21] When cyclosporin A was added to current medication in chronic severe oral glucocorticoid-dependent asthmatic subjects over a three month period there was an improvement in lung function and fewer disease exacerbations.[22] In a subsequent nine month study low dose oral cyclosporin A, but not placebo, significantly reduced the requirement for oral glucocorticoids and also produced a significant improvement in morning peak expiratory flow rates.[23]

Despite these observations it is not clear precisely how cyclosporin A ameliorates chronic asthma since, in addition to its inhibitory effects on the release of eosinophil-active cytokines from activated T lymphocytes,[24,25] it also inhibits other cellular functions including the rapid mediator release from mast cells in vitro and basophils ex vivo.[26,27] In order to dissect partially the possible effects of cyclosporin A on the late phase inflammatory and the early phase mast cell-mediated responses in vivo we have performed a randomised, double blind, placebo controlled, crossover study of the effects of oral cyclosporin A on the allergen-induced EAR and LAR in mild atopic asthmatic subjects. We hypothesised that cyclosporin A would inhibit both the EAR (through mast cell stabilisation) and the LAR (through inhibition of cytokine mRNA transcription by a wide variety of cell types).

Methods

PATIENTS

Twelve patients with atopic asthma were recruited from the allergy clinic of the Royal Brompton Hospital, London (table 1). The patients had a clinical history of intermittent chest tightness, wheeze or shortness of breath and documented reversible airflow obstruction (20% change in FEV_1) which occurred either spontaneously or with treatment in the preceding year. No subject was receiving long acting inhaled or oral β agonists. No subject had received immunotherapy or orally administered corticosteroids during the 12 months preceding the study. Four patients were taking inhaled corticosteroids but these were discontinued seven days prior to the allergen dose-finding visit. Patients with seasonal symptoms were studied out of the pollen season. None were smokers, and any patient with a history compatible with respiratory infection in the four weeks preceding or during the study was excluded. All patients gave written informed consent and the study was approved by the ethics committee of the hospital.

STUDY DESIGN

This was a double blind, placebo controlled, crossover study. The study period involved three inhaled allergen challenges with an interval of at least two weeks between each challenge. An initial challenge determined the allergen concentration sufficient to provoke a 20% reduction in FEV_1 from the prechallenge value (PC_{20}) within 15 minutes of allergen exposure (the EAR). Patients who developed an LAR (defined as a decrease in FEV_1 of >15% from baseline between 4–8 hours after challenge) were enrolled into the study. Patients received either two single doses of 500 mg cyclosporin A (in capsular form) or matched placebo before the two subsequent challenges in a predetermined random order. All other medication was withheld for at least eight hours before each allergen challenge.

At the initial assessment a full explanation of the study was given, the patient's history was taken and examination performed. Patients with any known contraindications to receiving cyclosporin A were excluded. Specific exclusion criteria were a previous or current history of gastrointestinal or liver disorders that could affect absorption, distribution, metabolism or excretion of the drug, as well as renal impairment as shown by one of the following: proteinuria (>0.3 g/l by dipstick analysis), serum creatinine >120 mmol/l, or concomitant treatment with nephrotoxic drugs. Patients with evidence of impaired liver function (any increases in serum bilirubin, aspartate aminotransferase, alkaline phosphatase or γ-glutamyl transferase more than twice the laboratory upper limits of normal) were also excluded, as were patients with a history of hypertension, cardiac disease, or epilepsy.

Skin prick tests to a panel of common aeroallergens (extracts of cat dander, dog fur, house dust mite, and timothy grass pollen) were performed, and blood was taken for an eosinophil count. The allergen used for challenge was selected on the basis of a history of clinical sensitivity supported by a positive skin prick test result (weal diameter at least 3 mm greater than that produced by control solution). The allergens selected for each subject are shown in table 1.

Allergen sensitivity was determined by skin prick tests with doubling serial dilutions of allergen, starting with 20 000 biological units (BU)/ml house dust mite (*Dermatophagoides pteronyssinus*), timothy grass extracts (*Phleum pratense*), or cat dander (ALK, Horsholm, Den-

mark). After challenge with inhaled saline to determine the baseline (pre-allergen) FEV_1, the dose-finding allergen challenge was performed, starting with a twofold dilution below that which produced a 3 mm diameter skin prick weal (threshold dose for a response). Increasing twofold concentration increments (with at least 15 minutes between challenges) were given until a 20% decrease in FEV_1 was achieved. Patients inhaled saline or allergen solution delivered by a Wright nebuliser (calibrated to give an output of 0.13 ml/min) by tidal breathing for two minutes. FEV_1 was recorded before challenge, every five minutes after allergen challenge for the first 30 minutes, and then half hourly for the next eight hours. At some time during the period 4–8 hours after the challenge patients were required to demonstrate a decrease in FEV_1 of at least 15% of the baseline value.

After a period of at least two weeks the patient received the first dose of study medication (either 500 mg cyclosporin A or placebo) at 20.00 hours on the evening before allergen challenge. At 08.00 hours the following day venous blood samples were taken for cyclosporin A concentrations and eosinophil counts before the patient received the second dose of the same study medication. At 10.45 hours, after further blood samples had been taken, a saline challenge was performed to determine the baseline FEV_1 (defined as the lowest FEV_1 value achieved within 15 minutes following saline challenge). The allergen challenge was performed at 11.00 hours using a predetermined dose of antigen (the PC_{20} allergen determined at the first visit). The FEV_1 was then recorded every five minutes for 30 minutes, then every 30 minutes for eight hours. Blood samples for further measurements of peripheral blood eosinophils were taken three, six, and 24 hours after allergen challenge and for measurement of cyclosporin A concentrations at 24 hours. The entire procedure was repeated after an interval of at least two weeks, with patients receiving the alternative treatment to that received on their first treatment visit.

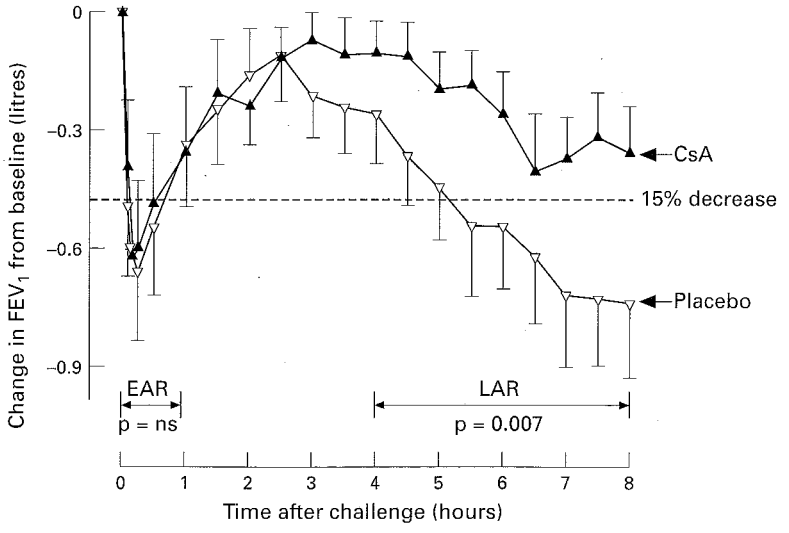

Figure 1 Effects of cyclosporin A (CsA) and placebo on mean (SE) changes in forced expiratory volume in one second (FEV_1) from baseline (prechallenge) values. The mean prechallenge FEV_1 values were 3.45 l for cyclosporin A and 3.44 l for placebo. The p values for individual comparisons of the effects of the two treatments on the areas under curve of changes in FEV_1 over the 0–1 hour and 4–8 hour periods are shown.

DATA ANALYSIS

The primary outcome measures were the magnitudes of the early (EAR) and late (LAR) asthmatic responses, defined as the area under curve (AUC) of changes in FEV_1 from the baseline (or prechallenge) value during the periods 0–1 hours and 4–8 hours after the challenge, respectively. The AUC values were calculated by the trapezoid method. Differences between the AUC values after cyclosporin A and placebo treatments were compared by paired analyses using a non-parametric method for crossover trials in which three separate Wilcoxon signed rank tests were performed to analyse the treatment and period effects as well as the treatment-period interaction.[28] This method was also used to compare absolute blood eosinophil counts at each time point. Correlation coefficients were calculated by Spearman's method with correction for tied values. Data analysis was performed by an independent blinded statistician.

Results

Every subject complied with the treatment, achieving blood cyclosporin concentrations of more than 100 mg/l 12 hours after the first dose of cyclosporin. Peak levels just before allergen challenge were more than 250 mg/l (mean (SE) 820 (130) mg/l). Nine of the 12 subjects reported some side effects (nausea or paraesthesia) after taking cyclosporin A while only one reported side effects after placebo treatment. All side effects were transient, lasting less than six hours, and were self-limiting.

FEV_1

FEV_1 measurements at each time point were found to be distributed normally but the AUC values for changes in FEV_1 from baseline were not. Comparison by a t test showed that baseline (pre-allergen) FEV_1 values during each treatment arm were not significantly different (mean (SE) 3.45 (0.15) l for cyclosporin A, 3.44 (0.16) l for placebo; $p = 0.69$).

The effects of cyclosporin A and placebo on changes in FEV_1 from baseline values after allergen challenge are shown in fig 1. There was no significant difference ($p = 0.63$) between the effects of cyclosporin A and placebo on the individual AUCs during the EAR period (0–1 hour after challenge). The median (and interquartile ranges (IQR)) of AUC values during this period were −12.0 (−36.9 to −7.0) litre hours (l.h) and −17.9 (−32.9 to −12.3) l.h after cyclosporin A and placebo, respectively. However, compared with placebo, cyclosporin A significantly reduced ($p = 0.007$) the AUC values during the 4–8 hour LAR period. The median AUCs were −41.9 (IQR −82.7 to −12.4) l.h for cyclosporin A and −84.5 (IQR −248.9 to −39.1) l.h for placebo. Compared

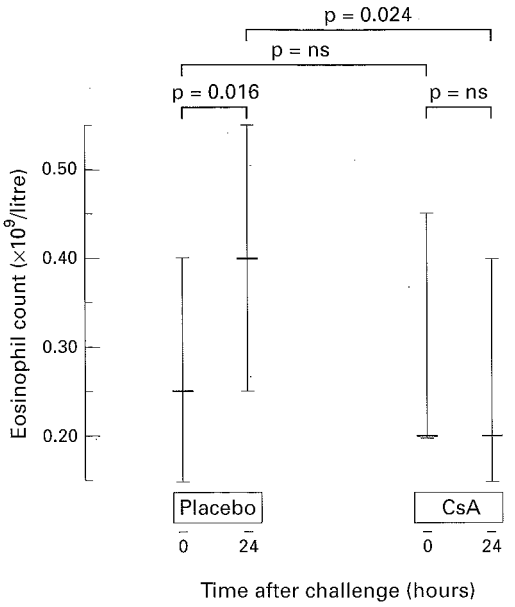

Figure 2 Effect of cyclosporin A (CsA) and placebo on the LAR associated increase in blood eosinophil counts 24 hours after allergen challenge (medians and interquartile ranges shown).

with placebo, cyclosporin A treatment was associated with a median reduction in the magnitude of the LAR of 65.9% (IQR 24.1 to 79.1%). There was a significant correlation between the percentage reduction in the magnitude of the LAR by cyclosporin A compared with placebo and blood cyclosporin A concentrations measured just prior to the allergen challenge ($r=0.6$, $p=0.028$).

EOSINOPHIL COUNTS

The baseline (prechallenge) absolute eosinophil counts were not significantly different for either treatment (median values 0.2 (IQR 0.2 to 0.45) $\times 10^9$/l for cyclosporin A and 0.25 (IQR 0.15 to 0.40) $\times 10^9$/l for placebo, $p=0.56$). By three hours after challenge there were small but significant reductions in blood eosinophil counts from baseline values with both treatment arms (median for cyclosporin A 0.15 (IQR 0.1 to 0.35) $\times 10^9$/l, $p=0.016$; median for placebo 0.2 (IQR 0.1 to 0.30) $\times 10^9$/l, $p=0.023$) but there were no significant differences between the two treatments ($p=0.94$). Similar reductions were observed at six hours (median eosinophil count for cyclosporin A 0.15 (IQR 0.1 to 0.35) $\times 10^9$/l, $p=0.031$ for changes from baseline; median for placebo 0.1 (IQR 0.1 to 0.30) $\times 10^9$/l, $p=0.008$) and again there was no significant difference between treatments ($p=0.52$). By 24 hours after allergen challenge, however, the eosinophil counts were not significantly different from baseline during treatment with cyclosporin A (median 0.2 $\times 10^9$/l, IQR 0.15 to 0.4, $p=0.80$) but were significantly increased during the placebo arm (median 0.4 $\times 10^9$/l, IQR 0.25 to 0.55, $p=0.016$). There was a significant difference ($p=0.024$) between the effects of the two treatments on eosinophil counts at 24 hours after challenge (fig 2).

There were no significant period effects or treatment-period interactions for either the eosinophil counts or AUC of changes in FEV_1.

Discussion

In this placebo controlled, double blind study we have shown that cyclosporin A modulated the late, but not the early, bronchoconstrictor reaction to inhaled allergen challenge of sensitised atopic asthmatic subjects. Furthermore, there was a significant correlation between the magnitude of the reduction of the LAR by cyclosporin A and the blood concentrations of cyclosporin A before the challenge. Cyclosporin A also abolished the increase in circulating eosinophils associated with the LAR 24 hours after allergen challenge but did not have any effect on early changes in blood eosinophil counts. Interestingly, the degree of inhibition of the LAR was similar to that observed with inhaled beclamethasone and sodium cromoglycate, drugs whose efficacy in the prophylaxis of asthma is well established.[12] The dose of cyclosporin A received by the patients as a single event was higher than that used for prolonged treatment in our previous studies on chronic asthma but was similar (on a mg/kg basis) to that given during induction of immunosuppressive therapy before organ transplantation. The objective was to ensure that a satisfactory blood concentration of cyclosporin A was rapidly achieved and then maintained during the period of maximal inflammatory activity following allergen challenge. This objective was accomplished in each patient.

The EAR is generally believed to result from bronchial smooth muscle contraction, vascular leakage, and mucosal oedema subsequent to the rapid release of pharmacological mediators such as histamine, leukotrienes C_4, D_4 and E_4, and prostaglandin D_2 (PGD_2) from IgE sensitised mast cells.[2,3] In contrast, the LAR is thought to be a reflection of bronchial mucosal inflammation.[1,4] Major cellular changes in the airways associated with the allergen induced LAR include increased numbers of activated CD4+ T lymphocytes and eosinophils.[6] The mechanism of eosinophil recruitment is complex but appears to involve the elaboration of CC chemokines (RANTES, MCP-3, MCP-4 and eotaxin) as well as the "eosinophil-active" cytokines interleukin (IL)-3, IL-5, and granulocyte/macrophage-colony stimulating factor (GM-CSF).[29,30] These cytokines promote eosinophil differentiation and maturation, enhance eosinophil adhesion and locomotion, and prolong eosinophil survival.[10] In particular, IL-5 is unique in its ability to differentiate the committed eosinophil precursor terminally. It also has hormone-like effects since in the guinea pig intravenous injection of IL-5 released mature eosinophils from the bone marrow.[31] Potential sources of eosinophil-active cytokines include T lymphocytes, mast cells, eosinophils, fibroblasts, endothelial and epithelial cells.[20,21,32–36] There is considerable circumstantial evidence that eosinophil-derived products are directly involved in the patho-

genesis of the LAR – for example, the magnitude of the allergen-induced LAR correlates closely with the numbers of activated eosinophils in bronchoalveolar lavage samples.[6]

Our observations of early reductions (within six hours of bronchial allergen challenge) of peripheral blood eosinophil counts are in line with previous studies[8 9 37] and may reflect early recruitment of eosinophils to the bronchial mucosa[38 39] as a result of the rapid release of mast cell derived cytokines and eosinophil chemoattractants such as TNF-α (which promotes transendothelial migration of eosinophils), leukotriene B_4, and platelet activating factor (PAF).[32] The possibility that this phenomenon reflected normal circadian variation was eliminated in a separate experiment (data not shown) in which no significant variations in circulating eosinophil numbers in these subjects were observed after bronchial challenge performed at the same time of day with saline alone. The eosinophils recruited to the airways are probably involved in the initiation of the changes associated with the LAR. The observation that cyclosporin A did not appear to affect this early recruitment of circulating eosinophils may help explain why it did not completely abolish the LAR. The late increase in blood eosinophil counts 24 hours after allergen inhalation mirrors that seen in the bronchi and may reflect increased cytokine driven differentiation of eosinophils in the bone marrow and subsequent release of mature eosinophils into the circulation.

Cyclosporin A has several anti-inflammatory effects which might be relevant to the observed attenuation of the allergen-induced LAR. Its actions were originally believed to be confined to CD4+ T lymphocytes where it inhibits allergen-induced cell activation and the transcription and translation of messenger RNA (mRNA) for several cytokines including IL-5 and GM-CSF.[24 25 40] It is now clear that it also affects cytokine production by other cell types including eosinophils, mast cells, and monocytes.[41–44] Cyclosporin A also modulates the rapid release of preformed and de novo synthesised mediators from human mast cells in vitro[26] and basophils ex vivo.[27]

Despite this wide array of biological activities in vitro or ex vivo it was notable that, whereas treatment with cyclosporin A modulated the LAR and the associated late increase in the peripheral blood eosinophil count in the present study, it had no effect on events believed to be associated with IgE mediated mast cell degranulation – that is, the EAR and the early reduction in circulating eosinophil numbers. The reasons for this are unclear. The pronounced effects on the LAR and the relationship between blood cyclosporin A levels and the magnitude of this reduction suggest that there was good bioavailability of the drug in the bronchial mucosa. Moreover, since the in vitro or ex vivo modulation of mast cell and basophil degranulation by cyclosporin A occurs within a matter of minutes after exposure to the drug,[26 27] the failure of cyclosporin A to inhibit the EAR is unlikely to reflect a latent period before the drug became effective.

The explanation most consistent with all of these observations is that, in this in vivo model, cyclosporin A exerted its anti-asthmatic effects mainly through inhibition of transcription and translation of cytokines in T lymphocytes, and possibly other cell types, rather than by significantly inhibiting release of mediators from mast cells. In this sense cyclosporin A can be considered to have anti-inflammatory as well as immunosuppressant properties. Taken together with the results of previous studies[22 23] these observations provide further indirect support for the involvement of eosinophil-active cytokines, of which allergen-specific T lymphocytes are believed to be important sources, in the pathogenesis of inflammation across the whole spectrum of asthma severity – from patients with mild asthma (such as those participating in the present study) to those with severe corticosteroid-dependent asthma. Although the use of oral cyclosporin A in mild asthma is unjustified because of its poor risk: benefit ratio, these observations suggest that safer agents which inhibit cytokine gene transcription might prove to be effective treatments for asthma without the potential side effects associated with the use of corticosteroids. An inhaled form of cyclosporin A offering the optimal combination of good local bioavailability and fewer systemic effects may be one such agent, particularly as the anti-inflammatory effects of cyclosporin A appeared to be dose related in this study. Indeed, inhaled cyclosporin A has already been shown to inhibit the LAR in an animal model.[45] Such novel therapeutic agents may hold exciting prospects for the future management of asthma.

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Chapter 5

Prolonged Immunotherapy And FcεRI Expression On Peripheral Blood Cells

5.1 Effects Of Maintenance Or Cessation Of Prolonged Allergen Specific Subcutaneous Injection Immunotherapy On Humoral Immune Responses And Cell Surface IgE Receptor Expression In Peripheral Blood

(unpublished paper)

Effects of Maintenance or Cessation of Prolonged Allergen Specific Subcutaneous Injection Immunotherapy on Humoral Immune Responses and Cell Surface IgE Receptor Expression in Peripheral Blood

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INTRODUCTION

The safety and efficacy of specific allergen injection immunotherapy (IT) in carefully selected patients with seasonal allergic rhinitis is now well established (1). Uncertainty still exists however as to the optimum duration of IT treatment and also whether IT has disease modifying potential. Moreover the underlying immunological mechanisms of IT are still unclear. Changes in several immunological parameters have been described in patients receiving IT but there is still debate as to the exact relevance of most of these changes to the observed symptomatic improvement (1-3).

Immunological changes in peripheral blood most consistently associated with allergen injection IT include attenuation of seasonal elevations in serum levels of allergen specific IgE (4) and, after several months of IT, a gradual decline in specific IgE levels (5). In contrast, concentrations of allergen specific IgG antibodies, so called 'blocking antibodies', tend to increase and have been associated with clinical improvement (6). Decreased basophil reactivity *in vitro* to IgE dependent stimuli (7), presumed to be reflected *in vivo* by reduced amounts of mast cell or basophil derived mediators in nasal secretions (8,9), has also been described.

IT affects cellular as well as humoral immune responses. IT has been shown to inhibit allergen-specific peripheral blood mononuclear cell proliferative responses (10,11). Successful allergen IT is associated with effective reductions in allergen-induced late phase reactions (LPRs) - both cutaneous (12) and nasal (13). Reductions in LPRs at both sites were associated with inhibition of infiltrating CD4+ T-lymphocytes and activated eosinophils and significantly increased expression of mRNA for interferon-y (IFN-γ) (12,13). The results of these and other studies have led to the hypothesis that successful IT may act by modifying the pattern of cytokines produced at sites of allergic inflammation from a predominantly T_H2 (e.g. interleukin-4, IL-4) towards a T_H1 pattern (e.g. IFN-γ) (14).

In a previously reported placebo controlled study of IT with Timothy grass pollen extract *(Phleum pratense,* Alutard SQ) in grass-pollen sensitive patients with severe seasonal rhinitis, significantly improved symptom scores and reduced medication requirements were observed in patients receiving IT (15). All participants in the original study then received active IT in open fashion for a further 3 years and symptomatic improvements continued to be demonstrated throughout the 3-4 years of IT. In addition, progressive and sustained reductions in pollen-induced cutaneous LPRs were observed during the treatment period although initial reductions in cutaneous early phase reactions (EPRs) were not maintained (16).

The study was continued further with 27 of the original patients (who had by then received continuous IT for 3-4 years) being randomised, in blinded manner, either to continue receiving active IT (ITA) or placebo injections (ITP) for a further 3 years. The results of this study have been reported elsewhere (17). The most striking findings were that no significant differences were observed in symptom scores, medication use and cutaneous late phase responses between both groups at the end of this study period. Notably improvements in all these parameters compared to baseline values had been maintained 6-7 years after initiation of IT in the ITA and ITP groups even though latter had not received active IT for 3 years.

In the present study we have investigated whether the maintenance or withdrawal of prolonged IT in these 27 subjects had any effect on particular immunological parameters in peripheral blood and have attempted to establish the possible relevance of any changes to the prolonged clinical efficacy of immunotherapy that was observed. Parameters specifically examined were serum total IgE (immunoglobulin E) concentrations, serum concentrations of allergen *(Phleum pratense)* specific IgE and IgG, FcεRI (the high affinity receptor for IgE) expression on basophils and monocytes and CD23 (the lower affinity receptor for IgE) expression on B-lymphocytes and serum concentrations of the soluble form of the α chain of FcεRI (sFcεRIα).

METHODS

Patients

The study was approved by the Ethics Committee of the Royal Brompton Hospital. Atopic patients were recruited from the Allergy Clinic at the Royal Brompton Hospital. The inclusion criteria for patients in both immunotherapy groups and the atopic control group (AC) were identical and have previously been described (15, 16). Briefly, these otherwise healthy patients all gave a history of severe seasonal rhinitis poorly controlled by conventional medication (anti-histamines, intranasal steroids and sodium cromoglycate) and all had a positive skin prick test (>5 mm wheal) to Timothy grass *(Phleum pratense,* Soluprick ALK, Denmark). Patients with chronic asthma were specifically excluded. The non-atopic controls (NC) had no symptoms suggestive of atopic disease and had negative skin-prick tests to a panel of 12 common aeroallergens. Full details of all four patient groups are given in table 1.

Study design

Twenty seven patients who had participated in the original study and had received either 3 or 4 years of immunotherapy continued to be assessed for a further 3 years for this study. In double-blind randomised

fashion 13 patients (the 'ITA' group) continued to receive maintenance allergen immunotherapy whilst 14 patients (the 'ITP' group) received a placebo preparation for their maintenance treatment. At the end of the study period, comparisons were made between the ITA and ITP groups and also two control groups containing 12 grass-pollen sensitive atopic rhinitics (AC) and 16 non -atopic subjects (NC).

Serum samples from the patients in the ITA and ITP were obtained at each summer and winter assessment visit during the 7 year study period (1989-1995) and stored at -80°C until the conclusion of the study when the concentrations of total IgE, specific IgE and specific IgG to Timothy grass pollen in these serum samples were determined. Additionally, FcεRIα expression on peripheral blood basophils and monocytes and expression of FcεRII (CD23) on B cells were assessed by flow-cytometry using unseparated blood samples obtained from these subjects at the conclusion of the study. Finally stored serum samples taken before and during the final rhinitis season studied (1995) were used to determine soluble FcεRIα concentrations (sFcεRIα) in both IT groups. All these blood and serum parameters (apart from sFcεRIα in the NC group) were also evaluated in each AC and NC subject during the winter and summer of the final season.

Flow-cytometry

The following directly-conjugated monoclonal antibodies (mAb) were used for two and three colour flow -cytometric analysis of the peripheral blood: CD20 fluorescein isothiocyanate (FITC) (Coulter), IgG1 FITC (Becton Dickinson), IgG2 isotypic control (Coulter), CD23 phycoerythrin (PE) (Coulter), CD14 PE (Becton Dickinson). 22E7 (isotype IgG1), a mAb specific for the α-chain of the high affinity receptor for the Fc portion of IgE, FcεRIα, was a kind gift from Drs. R. Chizzonite and J. Kochan, Hoffman-La Roche Inc., Nutley, NJ, USA. and was directly conjugated to FITC. The conjugation was performed by Cymbus Biotechnology Ltd, Hampshire, UK.

Aliquots of 100 µl of peripheral whole blood in EDTA were stained with the fluorochrome-conjugated antibodies and incubated in the dark at room temperature for 30 minutes. Red blood cells were then lysed and the remaining cells fixed using the 'Multiprep' system (Coulter). The cells were re-suspended in 250µl of 0.5% formaldehyde in an isotonic buffer (Isoton II, Coulter) prior to analysis on a Coulter Epics Elite flow cytometer and cell sorter (Coulter Corporation, Hialeah, FD). Identical gain settings were used for each sample and the cytometer flow cell was fully aligned and calibrated with Immuno-Check fluorospheres (Coulter) prior to each run. This allowed direct and valid inter- and intra-individual comparisons.

Monocytes were identified by a combination of CD14 expression and characteristic side-scatter and data from 5000 monocytes collected and analysed. Basophils were identified by their characteristic 22E7 staining and side-scatter appearances (as previously described (18)) and data from 1500-2000 basophils were collected and analysed. B-lymphocytes were identified by gating on CD20+ staining cells within the lymphocyte population (identifiable by forward and side-scatter). Data from 2000 B-lymphocytes were collected and analysed.

Cellular expression of FcεRI on basophils and monocytes was quantified by determining the specific mean fluorescence (smf). This was the difference between the mean fluorescence intensity (mfi) of cells stained with 22E7 and the mfi of the cells stained with an irrelevant isotypic control antibody.

Additionally, the percentage of monocytes expressing FcεRIα and B-lymphocytes expressing CD23 were analysed by the Immuno-4 software programme (Coulter). This programme used the Overton method of channel-by-channel histogram subtraction (19) to compare histograms generated by cells stained with the relevant anti-IgE receptor mAb and an identical number of cells stained with the isotype control mAb.

Serum total IgE, specific IgE and specific IgG measurements and sFcεRIα *assay*

Total IgE and *phleum pratense* specific concentrations of IgE were quantified by the UniCAP system (Pharmacia Diagnostics). Sera for quantification of allergen specific IgG and soluble FcεRIα were sent to the Laboratory of Medical Allergology, National University Hospital, Copenhagen. Specific IgG concentrations were measured by an enzyme linked immunosorbent assay and soluble FcεRIα levels were quantified by a sandwich immunoradiometric assay.

Statistical analysis

Data were analysed using a statistical package (Minitab for Windows, Minitab Release 9.2, Minitab Inc., State College, PA). Non-parametric analytical tests were used - Kruskal-Wallis ANOVAR analysis was used to assess variability within the study population and, if this was significant, the Mann-Whitney U test was used to analyse inter-group differences. For intra-group paired data comparisons the Wilcoxon signed ranks test was used. Correlation coefficients were calculated by Spearman's method with correction for tied values. For all tests, p values less than 0.05 were considered significant.

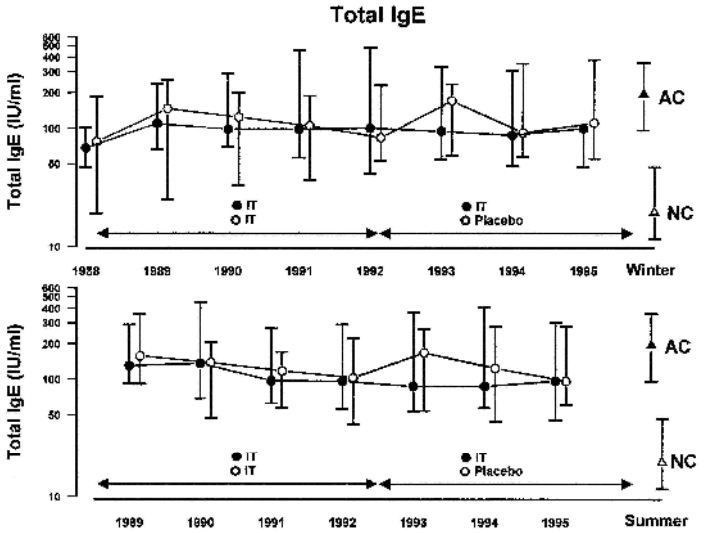
RESULTS

The group characteristics are summarised in table 1. The ITA and ITP subject groups were well matched with regard to total serum IgE concentrations when they commenced the study in 1989 (median values were 68 IU/ml for ITA, 77 IU/ml for ITP). Total IgE were also measured in the AC and NC control group subjects when they were recruited into the study in 1995. The IgE concentrations in the AC group did not differ significantly from the baseline measurements from the IT groups (median value 148.5 IU/ml) but concentrations in the NC group (median19.5 IU/ml) were significantly decreased when compared to the AC group (p= 0.02) and baseline values for the ITA group (p=0.03) but not the ITP group (p=0.08). There were no significant variations in serum total IgE levels in the ITA and the ITP groups in either winter or summer over the course of the study (figure 1).

Table 1: *Patient characteristics and baseline (1989 for ITA and ITP groups,1995 for control groups). serum total IgE and allergen specific IgE and IgG levels (median and interquartile ranges)*

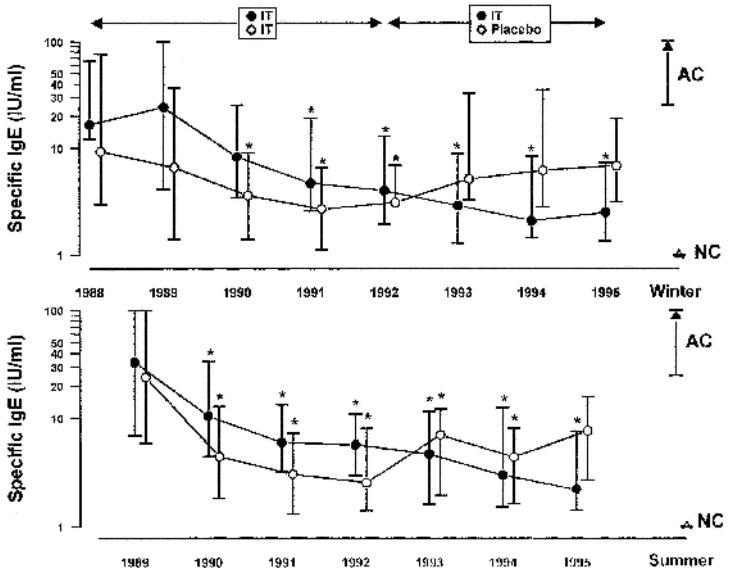
SUBJECT GROUPS	ITA	ITP	AC	NC
NUMBER	13	14	12	16
GENDER (m:f)	8:5	7:7	7:5	9:7
MEAN AGE (range)	41 y (19-65)	42 y (31-58)	36 y (22-56)	39 y (22-71)
TOTAL IgE (IU/ml)	68 (47-102)	77 (7-1020)	148.5 (80-576)	19.5 (11.5-46.5)
ALLERGEN SPECIFIC IgE (IU/ml)	16.6 (12.2-65.5)	9.3 (3-76)	32.2 (9.1-100)	0.0 (0.0-0.0)
ALLERGEN SPECIFIC IgG (IU/ml)	0.065 (0.053-0.08)	0.06 (0.045-0.085)	0.03 (0.02-0.043)	0.02 (0.0-0.02)

Figure 1:



Serum IgE levels in winter and summer months throughout the study period for both treatment groups (median and inter-quartile ranges). 1995 seasonal values for both control groups also shown.

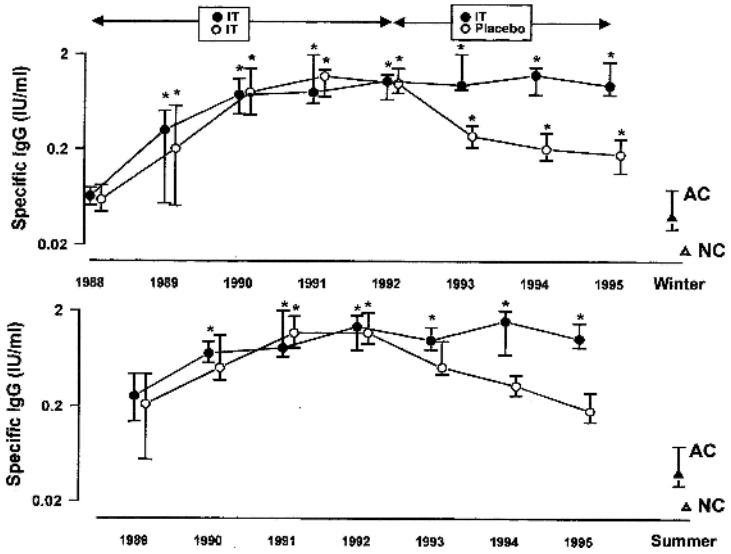
Figure 2:



*Allergen specific serum IgE levels in off peak (winter) and peak (summer) pollen seasons during the study. Final season values for both control groups also shown. * denotes values differ significantly ($p \leq 0.05$) from baseline values.*

There were no significant differences in allergen specific IgE concentrations at baseline between both IT groups. However after 3-4 years of immunotherapy allergen specific IgE concentrations were significantly decreased compared to baseline values in the ITA and ITP groups. This significant reduction continued in the ITA group over the entire follow-up period (figure 2). Although this effect was still evident in the ITP group in the peak season estimations for 2 years following cessation of IT, specific IgE concentrations had returned to baseline values by the third year after ceasing active immunotherapy. In contrast, allergen specific IgG concentrations in both winter and summer were significantly increased from baseline in both IT groups after 3-4 years of immunotherapy and these increases were maintained in the ITA group. In the ITP group specific IgG returned to baseline values in the summer, but not winter, assessments but continued to remain significantly increased when compared to the AC group (figure 3).

Figure 3:



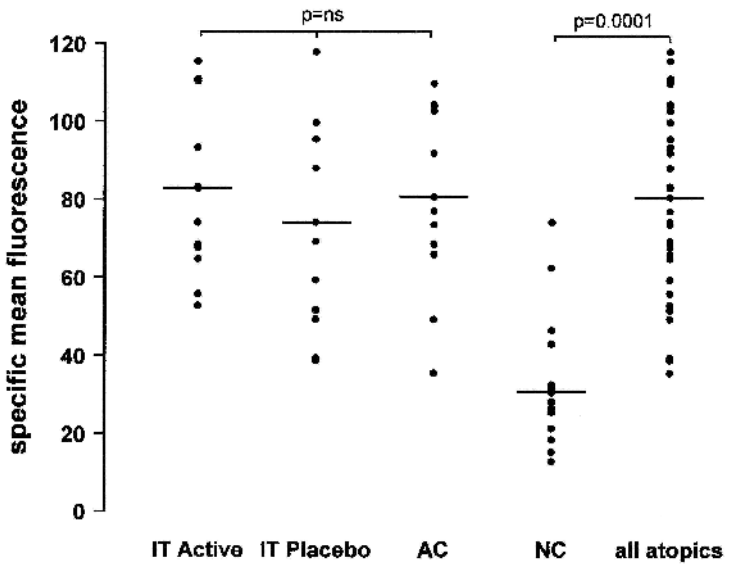
*Allergen specific IgG levels in serum in the ITA and ITP groups measured in winter and summer. * denotes values differ significantly ($p \leq 0.05$) from baseline values. Comparisons were also made with samples from atopic (AC) and non-atopic (NC) at the end of the study period.*

There were no significant seasonal variations observed in the serum concentrations of sFcεRIα in the ITP or AC groups but sFcεRIα in the ITA group were significantly elevated during peak season when compared to pre-season measurements (p=0.02) (table 2). However there were no significant inter-group differences in sFcεRIα levels, either pre- or peak season, between the three atopic groups.

Table 2: *Median values (and interquartile ranges) of CD4+ and CD8+ counts, CD23 expression on B lymphocytes, FcεRI expression on monocytes and soluble FcεRIα levels. * denotes values differ significantly ($p \leq 0.05$) from baseline values*

SUBJECT GROUPS	ITA	ITP	AC	NC
CD4 counts ($x10^9$/l)	0.88 (0.6-1.14)	0.92 (0.76-1.07)	0.98 (0.64-1.13)	0.81 (0.73-0.96)
CD8 counts ($x10^9$/l)	0.43 (0.23-0.51)	0.3 (0.25-0.43)	0.37 (0.31-0.4)	0.33 (0.28-0.49)
CD23+ B cells (%)	36.3 (19.4-62.1)	36.3 (10.2-64.7)	45.7 (20.2-82)	26.3 (18.2-41.3)
CD23+ B cells (smf)	111.1 (45.7-309.5)	105.7 (24.9-216.6)	142.9 (44.7-345.7)	78.9 (50.2-139.0)
FcεRI+ monocytes (%)	17.3 (14.4-30.8)	18.6 (8.0-34.2)	23.0 (15.5-28.8)	13.9 (9.2-23.4)
FcεRI+ monocytes (smf)	0.61 (0.38-1.26)	0.60 (0.30-1.0)	0.57 (0.15-0.89)	0.38 (0.20-0.9)
Pre-season sFcεRIα (pg/ml)	391 (286-773)	424 (324-607)	583 (458-987)	ND
Peak season sFcεRIα (pg/ml)	482 * (322-695)	437 (351-521)	455 (291-625)	ND

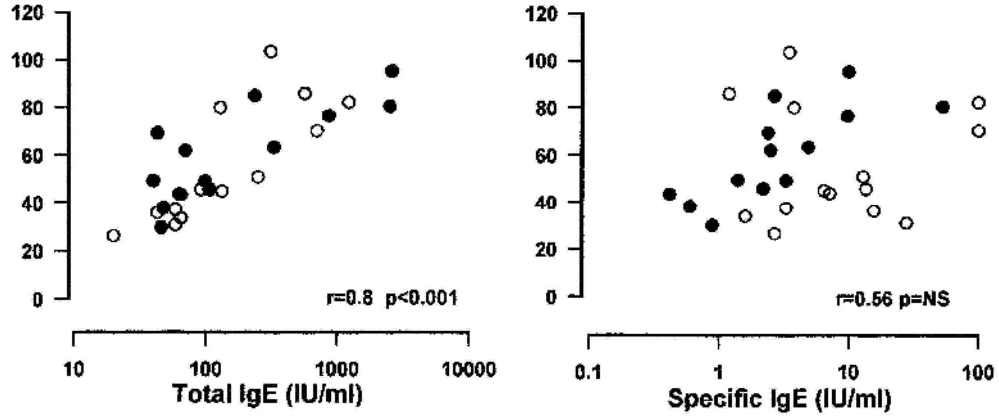
Figure 4:



Relative expression of FcεRIα on blood basophils (expressed as the specific mean fluorescence) in the 4 study groups

Figure 5:

a. b.



Graphs showing basophil FcεRI expression (represented by the specific mean fluorescence of staining with 22E7) in all 27 subjects who received IT against (a) their total serum IgE concentrations or (b) allergen specific serum IgE concentrations. Solid circles are subjects in ITA group and open circles are those from the ITP group.

There was no significant difference between basophil FcεRIα expression (measured as the specific mean fluorescence of staining with the 22E7 monoclonal antibody) in either of the group of subjects who had received prolonged immunotherapy (ITA and ITP) and the control atopic subjects. As anticipated however, basophil FcεRIα expression was significantly elevated in all three atopic groups when

compared to that in the normal control group (figure 4). There was a significant positive correlation ($r=0.8$, $p<0.001$) when basophil FcεRIα expression in subjects from both groups that received IT groups was compared to their serum total (but not allergen specific) IgE concentrations (figure 5). Interestingly, there was also a positive correlation between sFcεRIα and total IgE concentrations when the data from these 2 groups were similarly combined ($r=0.605$, $p=<0.01$) but only a weak positive correlation between sFcεRIα and allergen specific IgE concentrations ($r=0.45$, $p=0.02$). There were no significant differences between subjects receiving active or placebo IT in FcεRI expression on monocytes or FcεRII expression on B-lymphocytes.

SUMMARY

1. There were no significant variations in serum total IgE levels in the ITA and the ITP groups in either winter or summer over the course of the study ;
2. Serum allergen specific IgE was decreased in summer within the first year of commencing IT and remained significantly reduced whilst subjects continued to receive active IT. However summer allergen specific IgE levels no longer significantly differed from baseline 3 years after discontinuing active IT;
3. Allergen specific IgG concentrations in both winter and summer were significantly increased from baseline in whilst subjects received active IT. Allergen specific IgG returned to baseline values in the summer, but not winter, assessments when active IT was discontinued;
4. There were no significant changes in cellular FcεRIα expression on basophils and monocytes or FcεRII expression on B-cells;
5. Soluble FcεRIα levels did increase during the summer season in patients receiving active IT in the one year they were measured (the final year of the study).

This study and its results will be discussed in detail in Chapter 6, section 6.5.

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Chapter 6

Discussion

6.1 Prologue

The main body of this thesis has consisted of four papers (three published) describing original work that was done a few years ago. In the following sections, these studies will be put in context, with a discussion of how their observations and conclusions have been taken forward in subsequent years.

6.2 Studies Investigating Peripheral Blood Cells Expressing FcεRI

The paper in Chapter 2 (Sihra et al. 1997a) was the first published direct comparative study of FcεRI expression on basophils, monocytes and eosinophils, three blood cell types that may have potentially significant roles in initiating and maintaining allergic inflammation (discussed below). The study included 28 well characterised subjects with atopic disorders (asthma, rhinitis or dermatitis) as well as 10 non-atopic controls. The inclusion of such a large number of subjects allowed meaningful comparisons to be made between FcεRIα expression on these cell types and serum immunoglobulin E (IgE) concentrations. We were able to confirm the close linear correlation between basophil FcεRI expression and serum IgE concentrations first observed by Malveaux and colleagues (using indirect methodology) almost two decades previously (Malveaux et al 1978). Intriguingly there was also a less strong, but still significant, positive correlation between FcεRI expression on monocytes and serum IgE concentrations. The other main finding was that FcεRIα expression on eosinophils was detectable in only a few atopic subjects and, when these receptors were detectable, the level of expression much lower than on either of the other cell types.

The observations described in our paper were almost replicated subsequently in a similar study (Saini et al. 2000) performed at the Johns Hopkins Allergy and Asthma Centre by the group led by Donald W MacGlashan (which is a group that has been

prolific in investigating regulation of FcεRI expression on basophils). Their study used a very similar protocol to ours to examine FcεRIα expression on blood basophils, monocytes and eosinophils from subjects with a wider variety of conditions (non-atopic, allergic asthma, atopic dermatitis, hypereosinophilic syndromes, hyper IgE syndromes, helminth infestation or IgE myeloma) and also tried to correlate receptor expression with serum IgE levels. They too found a strong positive correlation between basophil FcεRIα surface expression and serum IgE concentrations ($r=0.67$, $p < 0.01$). However, although they observed higher expression of FcεRI on monocytes in atopics, they did not find a significant correlation between monocyte FcεRIα expression and serum IgE overall. They only detected eosinophil FcεRI expression in one subject with an IgE myeloma.

In the following sub-sections other studies that have been published subsequent to our paper and specifically took forward or expanded our observations of FcεRI expression on each of these three cell types are discussed.

6.2.1 Regulation of FcεRI expression on basophils

As has already been described, a close positive correlation has been observed between surface FcεRI expression and serum IgE levels. Furthermore it appears that the majority of receptors expressed on basophils are occupied by IgE molecules. As described in Section 2.2, receptor occupancy was estimated in our study from the observed intra-individual difference between the specific mean fluorescence intensity (smf) of receptor staining with two anti-FcεRIα mAbs (22E7, a non-competitive mAb that can be assumed to binds to all FcεRIα chains whether occupied or unoccupied by IgE and 15-1, a competitive mAb that binds only to unoccupied receptors). It was found that even in non-atopic subjects with low serum IgE concentrations, the majority of receptors (about 60%) appeared to be occupied. Furthermore receptor occupancy appeared to increase as serum IgE concentrations increased until receptor saturation was observed at serum IgE concentrations of greater than about 400 IU/ml.

Once again, just as with the correlation of FcεRI expression with serum IgE levels, these observations of increasing occupancy related to serum IgE with saturation

kinetics were strikingly similar to the observations described previously by Malveaux (Malveaux et al. 1978). Taken together, the observations that basophils from individuals with higher serum IgE concentrations tend not only to have greater numbers of FcεRI on their surfaces but also have a greater proportion of these surface receptors actually occupied by IgE would imply that the observed association between serum IgE and basophil FcεRI expression is not merely coincidental. One plausible explanation that would be consistent with all these observations would be that there is a causal relationship between serum IgE and surface FcεRI expression on circulating basophils – and this could perhaps result from receptor bound IgE somehow stabilising the receptors at cell surfaces.

Further evidence for the emerging concept that serum IgE could be a key modulator of cell membrane FcεRI expression on basophils became available at around this time from early clinical studies of a novel and exciting therapeutic agent – omalizumab (or, as it was known at that time, rhumAb-E25). This is a humanised, chimeric monoclonal anti-IgE antibody. As it binds to the Cε3 domain of IgE (the FcεRIα binding domain), it can only bind to free (i.e. not receptor bound) IgE. Therefore it is not anaphylactogenic (Presta et al. 1993;Saban et al. 1994). Unlike its binding to the FcεRIα chain, an IgE molecule can bind to two omalizumab molecules (and vice versa). In fact it has been demonstrated that this interaction can result in stable small (often cyclic hexamers), biologically inert complexes (Liu et al. 1995;Fox et al. 1996).

Early phase 1 studies demonstrated that serum free IgE levels fall rapidly to less than 10% of pre-infusion levels within minutes of an intravenous infusion of omalizumab, and that this reduction of free IgE levels is maintained with repeated regular infusions but gradual returns to baseline levels over a number of weeks after discontinuation of infusions (Fick 2002). The phase 1 studies therefore provided an ideal opportunity to assess the effects of changing IgE levels on basophil FcεRI expression.

In a study of 15 atopic patients who had been receiving regular infusions of rhumAb-E25 (omalizumab) for 3 months, MacGlashan et al observed that serum free IgE levels had decease to less than 1% of pre-treatment levels. They prepared enriched basophil preparations from peripheral blood and used flow cytometric analysis of

mean fluorescence intensity with 22E7 to analyse basophil FcεRIα expression. Further quantification of basophil receptor densities was done by measuring eluted total and BPO-specific IgE (after pre-saturating receptors *in vitro* with benylpenicilloyl (BPO)-specific IgE which can only bind to receptors unoccupied by IgE). The investigators found that the median number of FcεRI per basophil had decreased from 220000 to 8300 in their group of 15 subjects (Macglashan et al. 1997).

These subjects continued to receive rhumAb-E25 infusions for a total of 46 weeks and free IgE and basophil bound IgE levels, as well as basophil FcεRIα expression, were re-evaluated within six to eight weeks of discontinuation of infusions (Saini et al. 1999). Serum free IgE levels rose gradually by almost five-fold to around 16% of pre-treatment levels by eight weeks after discontinuation of rhumAb-E25 infusions. Specific mean fluorescence of basophil bound IgE and 22E7 binding, assessed by flow cytometry, increased in parallel with serum free IgE levels. Furthermore, using the quantitative technique described above, basophil FcεRI receptor numbers had returned to above 80% of pre-treatment levels. Finally, the investigators purified basophils from 11 of these subjects at the discontinuation of the infusions (i.e. with low levels of FcεRI expression) and cultured them *in vitro* for 7 days in the presence or absence of IgE. Analysis by flow cytometry demonstrated significantly increased cell bound IgE and FcεRIα expression on cells cultured in the presence of IgE (whereas there was decreased expression in those cultured without IgE).

Taken together, these studies quite elegantly demonstrated that the dramatic reductions in serum free IgE achieved by the ability of omalizumab to form bind with circulating IgE were associated with decreased basophil IgE and FcεRI expression and that when serum IgE levels were allowed to increase once more (by discontinuing omalizumab), FcεRI and cell bound IgE expression both increased again. These observations provided further supportive evidence that, at least on basophils, FcεRI expression seems to closely follow changes in IgE levels and therefore it is highly likely that IgE is the key regulator of FcεRI expression on these cells.

The obvious question that arose from these observations is "how does IgE regulate cell surface FcεRI expression?" There could be a number of possible mechanisms, including:

- Regulation of FcεRI synthesis by IgE (possibly involving regulation of gene transcription, stabilisation of transcripts or translation into protein of the various FcεRI constituent subunits);
- Regulation of intracellular processing and transport of FcεRI chains by IgE-mediated signalling;
- The binding of IgE by FcεRIα stabilises the receptor in the cell membrane.

Borkowski and her co-workers attempted to evaluate each of these hypotheses using two different cell lines in which they had transfected the cDNA of the subunits of the human FcεRI receptor. Neither of these cell lines were of mast cell or basophil lineage so the observations they made are not necessarily limited to either of these cell types. By disabling various parts of the intracellular machinery (such as signal transduction pathways, gene transcription promoters, intracellular protein transfer etc) in these cell lines they were able to assess the minimal requirements for IgE mediated regulation of surface FcεRI (Borkowski et al. 2001). They found that upregulation of surface FcεRI expression by IgE did not depend on intracellular signalling or increased gene transcription, mRNA expression for the three receptor chains or intracellular protein levels. Furthermore IgE mediated upregulation of surface FcεRI was similar in cell lines deficient of cDNA for the β-chain (i.e. upregulation of $\alpha\gamma_2$ receptors by IgE was as efficient as $\alpha\beta\gamma_2$ receptors in these transfected cell lines). They concluded that the minimal requirement for regulation of FcεRI on the cell surface is simply the degree of IgE binding by the α-chain and that the main mechanism underlying this appears to be the stabilisation of the receptor within the cell membrane by IgE, which prevents re-internalisation and degradation of the receptor.

They suggested quite a plausible model involving a continual turnover process of FcεRI between the membrane and a preformed intracellular receptor pool fed by a continued basal level of protein synthesis (which is not upregulated by IgE) and cytoplasmic degradation of some receptors. In this model, binding of IgE to surface

FcεRIα chains considerably slows down, if not stops, the process of internalisation of the receptor and therefore greatly increases the number of cell surface receptors.

The Borkowski study of course used transfected cell lines. MacGlashan et al attempted to answer the same question specifically in human basophils (MacGlashan et al. 2001). They used information derived from a series of experiments on enriched human basophil preparations as well as mathematical models to investigate whether IgE upregulates FcεRI expression by regulating synthesis or by preventing receptor loss from the cell membrane. In one experiment they reduced receptor occupancy by a very short lactic acid treatment. This only removed FcεRIα bound IgE. They found that removing receptor bound IgE resulted in accelerated loss only of the receptors that were now unoccupied, with no loss of occupied receptors. Moreover there was no receptor loss when there was 100% receptor occupancy by IgE. They also found that the presence or absence of IgE had no impact on mRNA expression for any of the three chains of FcεRI. Furthermore using pulse-labelling with ^{35}S methionine, they found that the rate of protein synthesis remained constant and was independent of the presence or absence of IgE. The conclusion of this complex study was similar to that of the Borkowski study i.e. they confirmed that FcεRIα surface expression on basophils is regulated by receptor bound IgE and that it appears to do this primarily by controlling the loss of receptors from the cell membrane with no impact on protein synthesis, which remains constant.

6.2.2 Expression and regulation of FcεRI on monocytes and monocyte derived dendritic cells in the blood

As discussed in Chapter 2.1, FcεRIα expression on monocytes in our group of atopic and non-atopic subjects showed a significant positive correlation to serum IgE levels. Furthermore, using the same method of calculating occupancy on monocytes as used for basophils (described in detail in Section 2.2), we observed that occupancy once again appeared to be related to serum IgE concentrations – albeit that this relationship was not quite as clearly obvious as for basophils – with receptor saturation appearing to occur at IgE levels of around 300-400 IU/ml and occupancy at low serum IgE concentrations seemingly much reduced in comparison to basophils. Once again, just

as with basophils, these observations raised the possibility that receptor bound IgE may regulate FcεRIα expression on the surface of monocytes.

A study by Reischl and colleagues also looking at FcεRI expression on monocytes from 58 non-atopic subjects was published at around the same time as our study (Reischl et al. 1996). They detected FcεRI on monocytes from all subjects (median 18% of FcεRI+ monocytes, range 2– 66%) but most of the receptors on these 'non-atopic' monocytes were unoccupied by IgE in contrast to monocytes from atopic subjects which showed higher receptor occupancy. The researchers then went on to examine whether they could stabilise or upregulate FcεRI expression on these 'non-atopic' monocytes. They found that FcεRIα expression was lost on cells cultured overnight in conventional culture medium in the absence of IgE but that overnight culture with IgE resulted in increased FcεRI expression and IgE binding. Interestingly culture in the presence of IL-4 had no effect on receptor expression.

This last finding of IL-4 not being effective at upregulating FcεRI expression contrasted with a study published some five years later (Gosset et al. 2001). In this study, blood monocytes from atopic asthmatics (some of whom had received glucocorticoid therapy) and from non-atopic controls were studied. Once again monocyte FcεRI expression was increased in the untreated atopic subjects compared to non-atopic controls – but of interest was the observation that FcεRI expression on monocytes in the group of atopics who had received steroid therapy was similar to that on the control subjects. The monocytes were than cultured *in vitro* in the presence or absence of IL-4, IL-13 or GM-CSF and it was found that there was a dose-dependent increase in FcεRIα chain expression on monocytes cultured with IL-4 or IL-13, but not GM-CSF, in both the atopic and non-atopic group. This was associated with an upregulation of α-chain but not γ-chain mRNA. These effects were inhibited by the presence of glucocorticoid. The findings of this study also contrasted with the basophil studies, which had not found enhanced FcεRI expression to be associated with cytokine induced upregulation of mRNA for any of the FcεRI chains. Therefore, although the presence of IgE once again seems to be important for monocyte FcεRI expression, it appears that the 'atopic state' (specifically the presence of the T_H2

cytokines IL-4 and probably IL-13) may well be an important extra factor modulating the expression of the receptor on APCs by upregulating synthesis of the α-chain.

High affinity Fc receptors on monocytes and other antigen presenting cells (APCs) differ from those on mast cells and basophils in that they lack the β-chain (Bieber 1996). Almost as soon as FcεRI were detected on monocytes, it was reported that they were functional – with signal transduction, increased intracellular Ca^{2+} and prostaglandin E2 release resulting from cross-linking receptors (Maurer et al. 1994;Takenaka et al. 1995). Moreover, and perhaps more significantly, it was demonstrated that the efficiency of allergen presentation to T-lymphocytes by monocytes was increased 100- to 1000-fold *in vitro* if the allergen was targeted to FcεRI on these cells via allergen-specific IgE (Maurer et al. 1995) – a concept known as antigen focusing. Other actions that have been shown to result from cross-linking of FcεRI on monocytes include induction of the transcription factor NF-κB (with subsequent synthesis and release of the pro-inflammatory cytokine TNF-α and the chemokine MCP-1) (Kraft et al. 2002) and prevention of monocyte apoptosis resulting from enhanced expression of the anti-apoptotic molecules bcl-2 and bcl-xl (Katoh et al. 2000).

Even given all these potential pro-inflammatory actions of FcεRI bearing monocytes, perhaps the most exciting development in the mid-nineties was the observation that blood monocytes are not necessarily terminally differentiated cells but can, given the right conditions, differentiate into the 'professional' antigen presenting cell, the dendritic cell (DC). Dendritic cells are probably the most potent inducers of primary antigen-specific immune response (Banchereau & Steinman 1998) but the relative paucity of these cells in tissues and their varying phenotype during their life cycle hampered study of their ontogeny and functions until the last decade, when it became apparent that at least one major subtype of DCs actually has a myelomonocytic lineage (reviewed by Peters et al (Peters et al. 1996)).

Several investigators observed that when monocytes are cultured *in vitro* in the presence of both IL-4 and GM-CSF, they terminally differentiate into cells that have the morphological and phenotypic characteristics of immature DCs (known as

monocyte derived DCs) (Kiertscher & Roth 1996;Pickl et al. 1996;Chapuis et al. 1997;Palucka et al. 1998). Cells possessing the phenotypic characteristics of these immature (or monocyte derived) DCs were also identified *in vivo* in the peripheral blood of normal and atopic subjects (Maurer et al. 1996) – in fact we too were also able to identify them with our flow cytometric technique involving 22E7 staining and side scatter (shown in figure 1a in Section 3.1) (Kon, Sihra et al. 1998). These cells differ from monocytes as CD14 expression is greatly reduced or absent but expression of MHC Class II molecules, such as HLA-DR and HLA-DQ, is greatly enhanced. Other markers include increased expression of CD1a, CD40 and CD86 – but, most significantly from the point of view of allergen focusing, they also have increased expression of FcεRI chains compared to monocytes (although less intensely so than basophils). Maurer et al showed that, as on other APCs, the FcεRI expressed on blood DCs are of the trimeric, $\alpha\gamma 2$, form (Maurer et al. 1996). They further observed that not only did these blood DCs demonstrate proficiency as stimulators of primary immune responses (as assessed by their capacity to induce proliferation of allogeneic naive CD4+ T-lymphocytes), but that they also enhanced secondary, FcεRI/IgE-dependent, allergen specific T-cell proliferation almost 10 times more efficiently than monocytes.

More recently it has become apparent that there are in fact two distinct subsets of dendritic cells in human blood (Robinson et al. 1999), together representing just about 0.3% of total blood leucocytes. These subsets are morphologically and phenotypically distinct and have been termed myeloid and plasmacytoid DCs (mDC and pDC). Immature mDCs are virtually identical to the monocyte derived DCs described above and have high levels of surface expression of myeloid markers such as the integrin CD11c and the C-type lectin CD207 as well as the co-stimulatory molecules CD40 and CD86. Plasmacytoid DCs, on the other hand, are morphologically similar to plasma cells and, in contrast to mDC, have no expression of CD11c but high expression of other markers such as CD123 (the α-chain of the interleukin-3 receptor) and, uniquely, a lectin recognised by a monoclonal antibody BDCA2 (Schuurhuis et al. 2006). Particularly in atopic subjects, both the mDC and pDC subsets in blood express FcεRI on their surfaces (Foster et al. 2003;Stary et al. 2005).

Both DC subsets also express different sets of Toll like receptors (TLR) – a family of highly conserved pattern recognition receptors (ten identified to date) that are crucial for innate immunity as they directly recognise conserved molecular patterns across different classes of pathogens and mediate cell activation (Kapsenberg 2003). Human mDCs express all TLR except TLR7 and TLR9, both of which are preferentially expressed on pDCs (Schuurhuis et al. 2006). In recent years, a great deal of interest has focused on trying to understand how antigenic ligation of these different types of TLRs on DCs sets in motion a sequence of events that can result in development of different antigen specific T-lymphocyte responses – either T_H1 or T_H2 immunity or regulatory T-cell responses (Kapsenberg 2003;Schuurhuis et al. 2006). It has become increasingly evident that whilst DCs are important sentinel cells, linking innate and adaptive immune responses, they are also pivotal in directing and modulating T-cell responses (Kapsenberg 2003;Abbas & Sharpe 2005;Novak & Bieber 2008). A brief synopsis of current understanding of this vital role regulatory role is provided in Section 6.6.

Most dendritic cells actually reside in tissues at sites in the body where pathogens may first be encountered – the skin, gut and respiratory tract. Several different tissue specific types of DC, both of myeloid and plasmacytoid lineage, have been identified (Novak et al. 2004) but it has become apparent that many of these DCs in the skin, nasal and oral mucosa as well as the lower respiratory tract are relatively immature myeloid lineage cells with high levels of surface FcεRI expression, particularly in atopic individuals (Semper et al. 1995;Allam et al. 2003;Stary et al. 2005;Allam et al. 2006).

Unfortunately characterisation of peripheral blood dendritic cells happened too late for them to be included in our study looking at the relationship between serum IgE and FcεRIα expression on peripheral blood cells (Section 2.1). In fact it was not until 2003 that substantive evidence was available of a strong positive linear correlation between FcεRI expression on both subsets of blood DCs and serum IgE levels (Foster et al. 2003). However prior to that there was indirect evidence suggesting such an association, including Maurer's observation of increased receptor expression on circulating DCs from atopic, but not non-atopic, donors (Maurer et al. 1996).

Furthermore, whilst the process of *in vitro* differentiation of DCs from monocytes (with IL-4 and GM-CSF) results in enhanced intracellular accumulation of FcεRIα chains in cells from all subjects, only cells from atopic donors seem to upregulate synthesis of the γ-chain, which is of course necessary for surface expression of the receptor (Novak et al. 2003). The ability of IL-4 to upregulate FcεRIα synthesis with increased intracellular accumulation in newly differentiated DCs has been confirmed by at least one other study (Geiger et al. 2000). Finally, another omalizumab study, this time in subjects with seasonal allergic rhinitis who received a six week course of infusions, provided conclusive evidence that, as for monocytes and basophils, free IgE does play an important role in modulating FcεRI expression on blood DCs as omalizumab induced decreases in serum IgE levels were associated with significantly decreased FcεRI expression on blood DCs (to a similar extent as decreases in basophil FcεRI expression) (Prussin et al. 2003).

In summary, therefore, there is now conclusive evidence that APCs in peripheral blood (specifically monocytes and immature dendritic cells, which are likely monocytes derived) can express the trimeric form of the FcεRI. However, in contrast to basophils (and mast cells), expression of the receptor is not constitutive but is inducible – with the number of cells expressing the receptor as well as cell surface density being increased in atopic individuals. It appears that IL-4 at least (and probably IL-13) is necessary for upregulation of synthesis of the α- and γ-chains in atopics. However it is likely that there is a recirculating process between the intracellular pool of the receptor and the cell membrane with IgE stabilising surface expression, just as in basophils.

6.2.3 FcεRI on eosinophils in allergy

The presence of high affinity IgE receptors on eosinophils was first described on cells obtained from patients with hypereosinophilic syndromes (Gounni et al. 1994b). By the time the paper presented in section 2.1 was published, FcεRIα immunoreactive eosinophils were being identified in tissue from several sites of allergic inflammation – including bronchial biopsies from asthmatics (Humbert et al. 1996) and bronchoalveolar lavage fluid after segmental allergen challenge (Rajakulasingam et

al. 1998), nasal mucosa after allergen induced rhinitis (Rajakulasingam et al. 1997) and in skin biopsies from subjects with allergen induced late phase cutaneous reactions (Barata et al. 1997). In the last study mentioned skin eosinophils also expressed mRNA for all three FcεRI subunits.

The contrast between the reported increased levels of FcεRIα expression in tissue eosinophils at sites of inflammation and that in blood eosinophils reported in our study led to three obvious questions:

1. Does the process of migration of circulating eosinophils into the extracellular matrix at inflamed sites and subsequent interaction with tissue factors such as cytokines and fibronectin result in a change of surface phenotype with upregulation of FcεRI expression in the cell membrane?
2. If tissue eosinophils do express surface receptors (albeit at very low levels when compared to basophils and even antigen presenting cells) are these receptors functional? If so what cellular actions do they contribute to?
3. Could it be that the techniques used to analyse tissue eosinophils (immunocytochemistry or Western blot analysis) were actually identifying FcεRIα chains stored intracellularly rather than surface proteins? Perhaps the observations in tissue cells were just artefactual and tissue eosinophils have no greater degree of surface FcεRI expression than blood eosinophils. Certainly, in a preliminary experiment for the study described below (Smith et al. 2000) in which we analysed blood eosinophils after their cell membranes were permeabilised by saponin (using the method described in Chapter 3), we found greatly enhanced binding of the 22E7 mAb even though the subjects had negligible surface expression of FcεRIα on their eosinophils (unpublished data).

In an attempt to address these questions a further study was set up by our group. I did the preliminary work leading up to the study and helped devise the study protocol but the main study was taken on and completed by Dr Susan Smith, a Post-doctoral Fellow in the Department (Smith et al. 2000). In this study eosinophils were purified from the peripheral blood of six atopic subjects. Baseline FcεRIα expression was assessed by flow cytometry as described in section 2.1. Intracellular expression of FcεRIα was also analysed by flow cytometry after the cell membrane was made

permeable with saponin. The cells were then cultured for 20 hours with a variety of factors that may possibly cause upregulation of eosinophil FcεRI surface expression in the microenvironment associated with allergic inflammation (IL-3, IL-4, IL-5, GM-CSF, fibronectin, tissue fibroblasts, IgE). As well as assessment by flow cytometry, cytospins were prepared pre- and post culture for subsequent analyses by in situ hybridisation (evaluating the expression of mRNA for each of the three FcεRI subunits) as well as immunocytochemistry.

Once again the blood eosinophils demonstrated negligible surface FcεRIα expression, smf (median and range) of 0.11 (0.10 – 0.12), thus confirming our previous observations, but after membrane permeabilisation there was increased 22E7 binding, smf 0.81 (0.41 – 1.0). This finding of increased expression of intracellular FcεRIα was also seen in cytospin preparations. None of the culture conditions resulted in increased surface expression of FcεRIα; nor did any of the conditions result in significant increases in mRNA expression for any of the three FcεRI chains (α, β or γ) – except for co-culture with fibroblasts resulting in a small but significant increase in FcεRIα mRNA (with no corresponding increase in the protein product). This study also tried to assess whether, even though the number of surface receptors detected were negligible in comparison to other cell types, they might have a functional role. It attempted to do this by evaluating whether there was any release of the eosinophil granule protein eosinophil peroxidase (EPO) resulting from crosslinking of receptors by an anti-FcεRIα mAb. There was no appreciable release of EPO resulting from cross-linking of FcεRIα.

The conclusions to this study were therefore that blood eosinophils express very low, (in fact, in comparison to basophils, almost negligible), numbers of cell surface FcεRI and that these receptors appear to be non-functional. Surface expression was not inducible by any of the culture conditions studied but there was evidence of a sizeable intracellular pool of preformed FcεRIα in eosinophils. Furthermore a large proportion of eosinophils appear to express mRNA for all three subunits of the FcεRI.

There have been three other studies published which have also attempted to try and answer some of these three questions (Seminario et al. 1999;Kita et al. 1999;Kayaba

et al. 2001). In a study performed by Seminario et al blood eosinophils were obtained from a variety of donors (normal controls, patients with asthma, allergic rhinitis or both and patients with a variety of hypereosinophilic disorders) were cultured for up to 11 days in the presence or absence of IgE and/or IL-4 (Seminario et al. 1999). The main findings of this study were similar to those of the Smith study – that is, no significant expression of surface FcεRI on eosinophils was observed at baseline or after culture. However there was evidence of a significant quantity of intracellular FcεRIα chains, and also FcεRIγ chains (but not β-chains). In contrast to their lack of surface α-chain expression, eosinophils did show surface expression of the γ-chain. Another interesting observation made in this study was that the supernatant of the cultured eosinophils did contain soluble FcεRIα chains. This indicated that the intracellular store of FcεRIα chains is available for release but for reasons that are unclear (certainly not apparently due to the lack of 'stabilising' γ-chains) very few, if any, α-chains get expressed on the cell surface.

The study by Kita et al (Kita et al. 1999) recognised that if eosinophils in the blood of atopic subjects express any FcεRI at all, the numbers are so few that even a sophisticated and sensitive technology like multi-colour flow cytometry may not be sufficient to detect them reliably. Therefore they used a different approach to try and detect and quantify them. They purified blood eosinophils from subjects with ragweed sensitive hayfever obtained at the peak of the hayfever season and, as usual, found no significant expression of surface FcεRI by flow cytometry. However they then cultured these cells with a chimeric IgE (cIgE) and confirmed that there was binding of cIgE to the cell surface. Since binding could be due to either (or both) high and low affinity Fc receptors (FcεRI and FcεRII) they looked at the impact of pre-treating the eosinophils with a saturating concentration of 15-1 (which of course is a competitive anti-FcεRIα mAb which would be expected to prevent IgE binding to the FcεRIα chain). Interestingly 15-1 did partially inhibit cIgE binding, suggesting that there was indeed FcεRI binding of IgE on these eosinophils. By quantifying the amount of 15-1 inihibitable cIgE binding on eosinophils in comparison to that on basophils and monocytes from the same subjects, the investigators were able to estimate the relative levels of FcεRI expression on these three cell types. The results were of a similar order of magnitude as in our study (section 2.1) – basophils FcεRIα expression was

some 125 times greater than monocytes and over 200 times more than eosinophils. However this study too was unable to detect any functional responses attributable to FcεRI on eosinophils – they found no IgE dependant degranulation or release of leukotriene C_4 or superoxide anion.

The final study was performed by the group that had originally described the presence of FcεRI in eosinophils from hypereosinophilic patients. Once again they concentrated mainly on subjects with hypereosinophilia or haematological malignancies, with only 6 normal and 5 atopic individuals in the group of 30 subjects (Kayaba et al. 2001). Nevertheless it is worth briefly summarising their findings. They applied a quantitative flow cytometric method to measure the expression of FcεRIα both on the cell surface and intracellularly. In this group of subjects as a whole they found that there were on average around 4500 FcεRIα chains per cell expressed on the surface (as a comparison, basophils from atopic subjects could be expected to express ≥ 200000 receptors per cell) but almost 50000 FcεRIα chains per eosinophil intracellularly. In this slightly atypical group of subjects there was, for the first time, a correlation noted between surface receptor of FcεRI on eosinophils and serum IgE concentrations. Their most interesting finding was that crosslinking of receptors with a murine anti-FcεRIα IgG mAb (15-1) together with the addition of an anti- mouse IgG polyclonal antibody resulted in the release of the cytokine IL-10. This therefore is the only study to have reported a functional response possibly attributable to FcεRI stimulation on eosinophils.

In summary therefore, a number of studies have confirmed our observation that if blood eosinophils express high affinity IgE Fc receptors at all, the level of expression is very low – probably no more than a couple of thousand per cell in atopic subjects. Furthermore it does not appear that surface receptor expression is inducible in tissues. However several studies have shown that eosinophils may have a sizeable intracellular store of preformed FcεRIα chains and it appears that these are releasable under certain circumstances. The significance of this observation is still unclear. Finally, there still remains uncertainty as to whether the few surface receptors that are present on eosinophils in atopic subjects have functional significance.

6.3 Basophils and Interleukin-4

Basophils comprise less than 1% of circulating leucocytes in humans. Although they were first described by Ehrlich over a century and a quarter ago, until recently they were considered to have little significance in immune responses (unlike their close cousins, mast cells and eosinophils). A major reason for this was that no basophil-specific markers had as yet been identified (and therefore there were no monoclonal antibodies, mAbs, with basophil specificity). This made it difficult to reliably identify basophils in blood or tissue and therefore greatly hindered research into their potential immunomodulatory roles.

It was only about a decade ago that researchers began to develop the specific tools necessary to take forward research into basophils and allergic inflammation. Technological advances included the development of highly sensitive fluorescence-activated flow cytometers and cell sorters with multi-colour detectors which allowed rapid purification of even small numbers of particles. This was the technology used in our study, described in Chapter 3, which made use of their unique phenotypic properties to positively identify circulating basophils and was one of the first to describe that basophils do have intracellular stores of the cytokine IL-4 which could potentially be rapidly released (Kon, Sihra et al.1998).

Further significant developments in subsequent years have included the availability, at last, of basophil specific mAbs. Two in particular, BB1 (a mAb which binds to basogranulin, a basophil granule specific component) and 2D7 (which also recognises a basophil granule protein), have enabled researchers to finally identify basophils in tissues at sites of allergen induced inflammation.

By using one of these mAbs to identify them in skin and bronchial biopsies following allergen induced late phase reactions, it was found that there is indeed an influx of basophils to these sites (Ying et al. 1999;Macfarlane et al. 2000;Nouri-Aria et al. 2001). In fact there appears to be almost a thirty-fold increase in basophil numbers at the site of cutaneous late phase reactions and an eight-fold increase in bronchial biopsies (Macfarlane et al. 2000;Nouri-Aria et al. 2001). Furthermore it was confirmed that, at least at sites of allergen induced asthma, basophils do store IL-4

protein – with 40% of the basophils identified staining positively for intracellular IL-4 (Nouri-Aria et al. 2001). Indeed, although the number of basophils in bronchial biopsies is still relatively low compared to eosinophils and mast cells (Macfarlane, et al. 2000), they are major sources of IL-4 (almost 20% of IL-4 mRNA positive cells after allergen challenge were basophils) (Nouri-Aria et al. 2001).

A significant difference between basophils and mast cells appears to be the repertoire of cytokines they can secrete. Whereas mast cells have been shown to have the ability to synthesise and secrete an extensive array of pro-inflammatory cytokines (such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, IFN-γ, TNF-α etc), basophils appear to be more specialised – only producing IL-4 and IL-13. However these cytokines are released in quite large quantities following IgE-dependant stimulation (Arock et al. 1993;Brunner et al. 1993;MacGlashan et al. 1994;Ochensberger et al. 1995;Gibbs et al. 1996;Li et al 1996) and, as has already been described, basophils appear to be major sources of IL-4 at sites of allergic inflammation. Furthermore, despite their relatively small numbers in blood, basophils were also found to be the predominant source of these two cytokines when peripheral blood mononuclear cells were stimulated by allergen *in vitro* (Devouassoux et al. 1999). The kinetics of the secretion from basophils are also important – as discussed in Chapter 3, preformed IL-4 has been shown to be rapidly released from basophils stimulated by anti-IgE antibodies and the time course for IL-13 secretion is also quite rapid (Gibbs et al. 1996).

The importance of the observations that basophils infiltrate sites of allergen-induced inflammation and are able to rapidly secrete these two particular cytokines (IL-4 and IL-13) has greatly changed the previous perception of them as having a rather limited role in allergy (perhaps just involved in immediate hypersensitivity) to possibly having important specialised roles in initiating and maintaining inflammatory responses. Both these cytokines are particularly significant as they work synergistically to help facilitate the influx of leucocytes (including eosinophils and basophils themselves). For instance, both promote upregulation of the adhesion molecule VCAM-1 on endothelial cells and are also potent inducers of the synthesis of the chemokine eotaxin by airway smooth muscle cells (Hirst et al. 2002;Moore et

al. 2002). Eotaxin is now considered to play a major role in the selective recruitment into lung tissue of eosinophils and basophils (both cell types express CCR3, the receptor for eotaxin) (Uguccioni et al. 1997;Ying et al. 1997;Conroy & Williams 2001). Basophil derived IL-4 and IL-13 may also play important roles in isotype switching of immunoglobulin synthesis to IgE (and IgG4), as has been demonstrated in an elegant experiment (Yanagihara et al. 1998) in which it was shown that IgE stimulated cultured basophils promoted B-lymphocyte proliferation and induced synthesis of IgE and IgG4 *in vitro* but these actions could be completely abrogated by the addition of neutralising mAbs to IL-4 and IL-13 to the culture. IL-4 is also particularly influential as it induces the development of the T_H2 phenotype of CD4+ T-lymphocytes – and the T_H2 pattern of regulatory cytokines (including IL-3, IL-4, IL-5 and IL-13) is particularly associated with the atopy (Abbas et al. 1996).

Despite *in vitro* data such as those discussed above supporting the hypothesis that basophils may have an important role in allergen induced T_H2 differentiation, *in vivo* confirmation has proved elusive until very recently (Min & Paul 2008). In a murine model, Sokol et al found that not only did the protease allergen papain directly stimulate basophils but, most notably, resulted in them being recruited to local lymph nodes where they secreted IL-4 as well as another T_H2 inducing cytokine TSLP (thymic stromal lymphopoetin) resulting in the differentiation of naive T-cells to T_H2 cells. Moreover they also observed that pre-treatment with an FcεRIα specific antibody that caused profound basophil depletion resulted in papain induced T_H2 responses being completely abolished – thus confirming the vital role that basophils play at least in protease allergen induced T_H2 responses in this model (Sokol et al. 2008).

One method of assessing the relative importance of the contribution of a pleiotropic cell to the processes of initiating and maintaining allergen induced inflammation is to assess the effectiveness of pharmacological agents that affect the cell's functions at inhibiting allergen provoked early and late phase reactions (EPR and LPR respectively). The mechanisms of such reactions (involving target organs such as the skin, nose or lungs) have been well characterised over the years. The EPR is believed to result primarily from the rapid release of preformed histamine and newly generated

lipid mediators such as cysteinyl leukotrienes and prostanoids from IgE sensitised mast cells and basophils (Casale et al. 1987;Liu et al. 1991). In contrast, the LPR involves a characteristic inflammatory response that includes increased T-lymphocyte activation, local eosinophil (and basophil) accumulation and increased production of T_H2 type cytokines (Bentley et al. 1997).

When the studies contributing to this dissertation were performed, there were no agents available that had been shown to inhibit basophil function specifically. However the immunosuppressive drug cyclosporin A had been shown to be effective in patients with chronic, severe corticosteroid dependant asthma (Alexander et al. 1992;Lock et al. 1996). CsA was initially thought to act primarily by inhibiting T-lymphocyte function, but a number of studies had shown that it has a greater array of actions, including effective inhibition *in vitro* of IgE mediated exocytosis from basophils and mast cells (and subsequently it has also been shown to inhibit IL-4 and IL-13 synthesis in basophils) (Triggiani et al. 1989;Casolaro et al. 1993;Patella et al. 1998;Genovese et al. 2003). In the study described in Chapter 4, the effects of CsA on allergen provoked early and late asthmatic reactions were assessed and discussed. The discussion in Section 6.4 focuses on the contributions made both by our study and an ensuing one that also evaluated the effects of CsA on allergen induced asthmatic reactions to increased understanding of the roles that basophils as well as eosinophils play in these reactions.

6.4 The Effects Of Cyclosporin A On Allergen-Induced Early And Late Phase Asthmatic Reactions

The main findings of the double blind, placebo controlled cross-over study described in Chapter 4 (Sihra et al. 1997b) were that cyclosporin A (CsA) significantly attenuated the late phase allergen induced asthmatic reaction and the associated increase in blood eosinophil counts but had no discernable effect on the early phase asthmatic reaction (which, as discussed above, is thought to be due mainly to allergen induced degranulation and release of mediators, such as histamine and cysteinyl leukotrienes, from IgE bearing mast cells and basophils in the airways).

Following the completion of the study in Chapter 4, there has been one further study of CsA and allergen induced asthmatic reactions. That study by Khan et al, published in 2000, was once again a double blind, placebo controlled randomised study involving pre-dosing of subjects with one dose of CSA or placebo before allergen challenge. However there were two differences in study design – it was a parallel group rather than a cross-over trial and on this occasion the subjects underwent bronchoscopies 24 hours before and after their allergen challenges (Khan et al. 2000).

The aims of this second study were, firstly, to assess whether the observations of our study of the differential effects of CsA on early and late phase asthmatic reactions were reproducible but, more particularly, to determine whether CsA does have the inhibitory effects on eosinophil influx and eosinophil-active cytokines in the bronchi that were predicted in our study. A further aspect of this study was the availability of the basophil specific monoclonal antibody BB1. This allowed assessment of the possible impact of CsA on basophil influx as well.

The results were interesting. Once again the late, but not the early, asthmatic reaction was significantly attenuated by CsA (confirming that CsA does not have a major impact on allergen induced, FcεRI mediated rapid degranulation of airway associated mast cells and basophils). CsA also significantly inhibited allergen induced increases in the number of activated eosinophils and cells secreting eotaxin in bronchial biopsies. Furthermore CsA also significantly inhibited allergen induced increases in cells expressing mRNA for the eosinophil-active cytokines IL-5 and GM-CSF studied in bronchoalveolar lavage fluid. Somewhat counterintuitively however, CsA did not have any significant impact on the allergen induced increases of basophils observed in the bronchial mucosa.

The authors' conclusions were very similar to the conclusions of our original study – that the attenuation of the late asthmatic reaction by CsA appears to be related to its inhibitory effects on synthesis of eosinophil-active cytokines and chemokines. However they did not really discuss the unexpected lack of impact of CsA on basophil influx. Basophils and eosinophils both express large numbers of the eotaxin receptor CCR3 and eotaxin is a strong chemoattractant for basophils, as it is for eosinophils

(Uguccioni et al. 1997;Iikura et al. 2001). Furthermore other chemokines that are involved in eosinophil chemotaxis, including RANTES and MCP-4 (acting via the receptor CCR3) also act on basophils (Uguccioni et al. 1997). Therefore it would be expected that the CsA associated effects on eotaxin and eosinophil influx would be mirrored by a similar impact on basophil influx.

A clue may perhaps be obtained from observations of the kinetics of cell recruitment to the sites of allergen induced late phase reactions in the skin. As the skin is more readily accessible than the bronchi, it is feasible to perform biopsies at more frequent time points than in the lung. In one such study of kinetics (Macfarlane et al. 2000) it was observed that whilst eosinophil influx was relatively rapid (peaking by six hours after allergen challenge), basophil influx was slower (peaking by twenty four hours after challenge). It seems probable that a similar difference in the rate of infiltration of basophils and eosinophils to the lung might be observed after allergen challenge if it were possible to take samples at additional time points.

There could be several possible explanations for a difference in the kinetics of infiltration:

- Whilst both cell types respond to chemokines acting through the CCR3 receptors (eotaxin, RANTES, MCP-4), these chemokines could act differentially (or have a different sequence of actions) on these two different cell types. There is *in vitro* data from at least one study that may support such a hypothesis (Heinemann et al. 2000).
- There may be other basophil specific chemokines or modulator factors (such as other cytokines). Basophils express five other types of transmembrane chemokine receptors besides CCR3 (CCR1, CCR2, CXCR1, CXCR2, CXCR4) and therefore do have the potential to respond to other chemokines – for instance IL-8 and MCP-1, which act via CXCR1 and CCR2 respectively can induce migration of basophils *in vitro* (but they do not exhibit the same degree of potency as eotaxin) (Iikura et al. 2001). More promisingly, insulin-like growth factors-1 and -2 (IGF-1, IGF-2) have been shown to show selective basophil chemotaxis and enhance the chemotactic effects of eotaxin (Hartnell et al. 2004).

- The presence of blood-derived granulocytes in tissues is a dynamic process dependant on the rate of influx into the target organ and survival of the cells when they are in the extravascular micro-environment. Biopsies at any time point do, of course, represent just a snapshot of these dynamic processes. Relatively little is known about the lifespan of basophils in tissues, with estimates varying from a few hours to a few days. A recent study found that *in vitro*, almost 60% of purified basophils underwent apoptosis by twenty four hours but that co-culture with IL-3 significantly prolonged basophil survival (Zheng et al. 2002). This finding was consistent with a previous report that IL-3 is the main cytokine promoting basophil survival with other cytokines such as GM-CSF having relatively little efficacy (Yamaguchi et al. 1992). This of course contrasts with eosinophils where IL-3, IL-5 and GM-CSF all enhance survival.

Whilst the study by Khan et al evaluated the effect of CsA on IL-5 and GM-CSF, it did not report on its impact, if any, on IL-3 synthesis nor did it look at its effects on chemotactic factors other than eotaxin which may regulate basophil trafficking. Moreover there did not appear to have been any evaluation of possible effects of CsA on basophil activation (e.g. analysis of CD69 expression). Taking all these variables into consideration the apparent dichotomous effects of CsA on eosinophil and basophil numbers in bronchial biopsies taken twenty four hours after allergen challenge may no longer appear to be quite so paradoxical.

Furthermore whilst the Khan study confirmed our observation of the lack of any significant impact of CsA on basophil or mast cell exocytosis (which is a relatively 'upstream' events in signal transduction, as discussed in Section 1.5.3), the study did not look at its impact on IL-4 or IL-13 production. As discussed in Chapter 3 and in Section 6.3, basophils appear to be prominent sources of both IL-4 and IL-13, cytokines that may make significant contributions to the initiation and maintenance of IgE dependant allergen induced late phase allergic reactions. It is possible, given the known effects of CsA on downstream signalling events, that even though CsA does not appear to have any effect on basophil influx, it may still impact on their function by inhibiting cytokine synthesis. In fact, although there have not been any published studies investigating possible effect of CsA on allergen induced basophil cytokine

production, effective *in vitro* inhibition of IgE/FcεRI mediated basophil cytokine synthesis by CsA has been demonstrated by at least two studies. In the first study, CsA inhibited endogenous superantigen induced IL-4 release from basophils (Patella et al. 1998) and in the second it inhibited IL-4 and IL-13 secretion from basophils stimulated by a bacterial superantigen (Genovese et al. 2003). Both these superantigens were shown to act on basophils by crosslinking FcεRI bound IgE.

In conclusion, both studies looking at the effects of CsA on allergen induced reactions have demonstrated that CsA is effective in inhibiting the late asthmatic reaction but not the early asthmatic reaction (which is associated with exocytosis of preformed mediators such as histamine from FcεRI bearing cells such as mast cells and basophils). Data from our study suggested that the effective inhibition of the late response may be due to inhibitory effects of CsA on eosinophil-active cytokines such as IL-5 and GM-CSF. The subsequent study appeared to support this hypothesis with significant impact on activated eosinophils in bronchial mucosa as well as IL-5 and GM-CSF. Interestingly CsA did not appear to have any impact on the increase in bronchial basophils observed twenty four hours after challenge. Whilst initially this may appear to indicate that basophils do not play a significant role in late asthmatic reactions, from the above discussion it is clear that there are still a lot of unanswered questions – and that as well as its effects on eosinophil influx and activation, the possible inhibitory effects of CsA on cytokine synthesis in basophils may quite conceivably make a significant contribution to its efficacy.

6.5 Prolonged Grass Pollen Immunotherapy And FcεRI Expression On Peripheral Blood Cells

The efficacy of allergen specific injection immunotherapy (IT) in selected patients with seasonal allergic rhinitis is by now well established and many of the underlying mechanisms are quite well understood (Durham & Till 1998;Till et al. 2004). The study described in Chapter 5 (Durham et al. 1999;Sihra et al. 2000) was unusual in that it compared two groups of subjects who had successfully achieved symptomatic remission after prolonged grass pollen immunotherapy (up to four years) and then, in a double blind fashion, assessed whether the remission was sustained up to three years later, whether or not the subjects continued with active or placebo injections.

It has already been reported that subjects participating in this study did indeed maintain their clinical improvements and decreased magnitudes of the allergen-induced cutaneous late (but not early) phase reactions to Timothy grass pollen (*Phleum pratense*) for at least three years after prolonged IT had been withdrawn (with no significant differences between the patients who continued to receive immunotherapy, the ITA group, and those who did not, the ITP group) (Durham et al. 1999). Of interest however were changes observed in various immunological markers in peripheral blood. Consideration of the patterns of these changes could have helped elucidate if any the changes associated with active immunotherapy remain relevant to the prolonged efficacy of immunotherapy even after it is discontinued.

Whereas total circulating IgE concentrations remained unaltered for the duration of the study, immunotherapy was associated with significant changes in allergen specific immunoglobulins - IgE levels were reduced and IgG levels became elevated. These changes were apparent both in and out of the pollen season, with no seasonal variations observed. However, although these alterations were maintained in the group continuing to receive immunotherapy (ITA), withdrawal of immunotherapy in the ITP group of subjects was associated with allergen specific immunoglobulin concentrations tending to return towards pre-treatment levels in spite of their sustained clinical improvement.

Although pre-immunotherapy data on cellular surface expression of IgE receptors or serum concentrations of the soluble portion of the α-chain of the high affinity IgE receptor (sFcεRIα) were unavailable, we were able to compare both immunotherapy groups (ITA and ITP) as well as 2 control groups (untreated rhinitics and non-atopic controls) at the end of the study. It was notable that at the conclusion of the study the surface expression of high affinity IgE receptors on both basophils and monocytes and also low affinity receptors on B-lymphocytes was similar in each of the three atopic groups irrespective of whether or not they had received immunotherapy. Furthermore although sFcεRIα levels measured in the final study year appeared to be significantly elevated during the pollen season (compared to pre-season values) in the ITA group, sFcεRIα levels did not significantly vary between the three atopic groups (ITA, ITP

and atopic controls).

The relevance of changes in specific immunoglobulins observed during IT has long been debated (Flicker & Valenta 2003). The 'blocking antibody' hypothesis suggests that elevated levels of allergen-specific IgG antibodies sequester allergen, preventing cross-linking of cell surface bound allergen-specific IgE and preventing IgE dependent cellular effector actions (van Neerven et al. 2006). Certainly there is *in vitro* evidence suggesting IgG blocking activity can prevent mediator release (van Ree & Aalberse 1995) and some studies have observed an association between specific IgG levels and clinical response to IT (McHugh et al. 1990). Certainly the initiation of allergen specific immunotherapy is associated with elevated serum concentrations of allergen specific IgG antibodies (Gehlhar et al. 1999;Till et al. 2004). However the results of the present study indicate that clinical improvements were maintained after withdrawal of prolonged IT even though specific IgE and IgG levels returned to pre-treatment values in the group of subjects receiving the placebo treatment (although they continued to remain significantly altered in those subjects continuing to receive active immunotherapy). This could possibly indicate that changes in specific immunoglobulins observed after prolonged IT may just be 'bystander phenomena', reflecting altered immunoglobulin isotype switching responses, and are not directly relevant to sustaining the clinical benefit of IT after it has been withdrawn. Another possible explanation is that measurement of serum levels is a rather crude way of evaluating the relevance of blocking antibodies – but functional assays that measure the blocking activity of allergen specific IgG, particularly of the IgG4 subclass (Nouri-Aria et al. 2004), may provide more clinically relevant data (Larche et al. 2006).

The low affinity Fc receptor for IgE (FcεRII or CD23) is a C-type lectin that binds IgE with a lower affinity than FcεRI (Bonnefoy et al. 1993a). CD23 molecules expressed on B-lymphocytes appear to have several functions which may be important in the IgE mediated inflammatory cascade (summarised in Section 1.6). IL-4 of course plays an important role in the preferential up-regulation of CD23 expression on B-lymphocytes (a role that is significantly inhibited by IFN-γ) (Bonnefoy et al. 1993a; Lee et al. 1993).

It may be surmised therefore that effective allergen immunotherapy would result in reduced expression of CD23 on B-lymphocytes. Indeed, significant reductions in both the percentage of CD23 expressing B-cells and receptor density have been observed in a group of asthmatic subjects who received rush immunotherapy with *Dermatophagoides pteronyssinus*, (Kljaic-Turkalj et al. 1996). However the results of our study did not show any significant reductions in CD23 expression after prolonged IT. One possible reason could be that the freshly isolated B-cells were analysed in a resting state as they were studied at the end of the pollen season and it is possible that the findings might have been different had these B-lymphocytes obtained from these subjects who had undergone prolonged immunotherapy been co-cultured *in vitro* with grass pollen (*Phleum pratense*). Indeed reduced expression of CD23 on allergen-stimulated cultured peripheral blood B cells following immunotherapy has been reported in at least one study (Jung et al. 1995).

As has already been discussed in the papers comprising Chapters 1 and 2, high affinity Fc receptors for IgE (FcεRI) are expressed not just on basophils and mast cells but also on other effector cells - including blood monocytes, on which FcεRI expression is up-regulated in atopics (Maurer et al 1994;Sihra et al 1997a), and monocytes-derived dendritic cells (Maurer et al. 1996). Furthermore, as described in Section 5.2, FcεRI on such antigen presenting cells (APC) appear to lack the β subunit (Bieber 1996)and are therefore expressed as trimeric complexes in contrast to classical FcεRI expressed on cells containing preformed mediators (mast cells, basophils and even eosinophils), which are tetrameric complexes.

Immunotherapy could potentially exert several immunomodulatory effects through actions on FcεRI expressing cells. As has already been discussed, both mast cells and basophils have been shown to be sources of T_H2-type cytokines (particularly IL-4) which can be rapidly released following IgE-dependent stimulation (Bradding 1996;Gibbs et al. 1996). Thus these cells may not just have roles limited to the allergen induced early phase reaction (EPR) but may also be involved in initiating the inflammatory changes associated with late phase reactions (LPR). The critical role of IL-4 in promoting IgE isotype switching has been discussed above. Effective

immunotherapy is associated with reduced IgE-dependent basophil releasability of preformed mediators (Malling et al. 1982). Suggested mechanisms for this include either reduced FcεRI surface expression or functional impairments of these receptors (Kimura et al. 1985). Although no study has as yet specifically assessed this, it is also likely that basophil cytokine releasability is significantly impaired by IT.

Potentially of even greater significance is the expression of FcεRI on professional APC such as monocytes. The presence of allergen-specific IgE on these receptors 'focuses' allergen presentation to T-lymphocytes (similar to the role of IgE/FcεRII on B-cells). Such FcεRI dependent allergen focusing has been shown to increase the effectiveness of antigen presentation by monocytes by up to 1000-fold *in vitro* (Maurer et al. 1995). It is possible that enhanced allergen presentation is important particularly at the initiation of immunotherapy as elevated allergen concentrations may promote the T_H1 phenotype (Hosken et al. 1995) and in fact have been shown to significantly reduce *in vitro* IL-4 production by CD4+ T-cells, particularly when allergen is presented by monocytes (Secrist et al. 1995). However it is also feasible that prolonged IT could induce decreased IgE-dependent allergen trapping by APC and therefore less efficient antigen presentation to T-cells - reflected *in vitro* by reduced allergen-specific proliferative responses (Baskar et al. 1997;Ebner et al. 1997). As well as possibly regulating the functions of cell membrane FcεRI, IT could conceivably act by actually down-regulating surface expression of these receptors both on granular cells and on monocytes, as IL-4 has been shown *in vitro* to up-regulate FcεRI expression on mast cells (Toru et al. 1996) and monocytes (Reischl et al. 1997).

However in the present study we were unable to demonstrate significant reductions in surface FcεRl expression on blood basophils or monocytes following IT. In retrospect this should perhaps not be surprising, since we now know that FcεRI expression on these cell types *in vivo* appears to be related predominantly to serum total IgE concentrations (Sihra et al. 1997a), and these were not affected by IT. Indeed it was interesting that there was still a significant positive correlation between basophil FcεRI expression and circulating total (but not specific) IgE concentrations after IT in our study group. It would certainly be of interest to evaluate the amount of allergen

specific IgE actually bound to these receptors as well as the IgE-dependent allergen presenting capacity of monocytes from IT treated subjects.

A soluble form of the α chain of FcεR1 (sFcεRIα) that appears to be stored in, and released, from the granules of basophils and eosinophils and retains the high affinity IgE binding capability of membrane-bound IgE (Bjerke et al. 1994;Bjerke et al. 1997) has been identified. Although its exact significance is unclear, an inhibitory role has been postulated for this molecule in IgE-mediated allergic inflammation. Recombinant sFcεRlα reportedly inhibited IL-4 and allergen-induced IgE production selectively *in vitro* (Bjerke et al. 1994;Yanagihara et al. 1994) – possibly by binding to CD23-bound IgE on B-lymphocytes and thus blocking allergen binding. As sFcεRlα is monovalent, it is non-anaphylactogenic – in fact the recombinant form appears to prevent mast cell degranulation *in vivo* (Naito et al. 1996). Thus sFcεRIα could theoretically be another natural 'blocking' molecule in allergy. The mechanisms modulating sFcεRIα levels have still not been established. Therefore the observation in this study of a positive correlation to total IgE concentrations in IT treated patients (rather similar to that observed between basophil FcεRI expression and total IgE) is intriguing. In the context of the mechanisms of IT, of more interest was the observation of elevated sFcεRIα levels during the pollen season in the group receiving active IT. This observation was somewhat counterbalanced overall by the lack of significant differences between the ITA and ITP groups. However it does suggest that further analyses of the role of this molecule, particularly during the early course of IT, would be merited.

In summary, the results of this study suggested that the clinical efficacy of 3-4 years of continued allergen injection immunotherapy may be sustained for at least a few years after cessation of treatment. However this was not reflected by significant quantitative changes in any of the blood parameters we looked at (humoral factors or IgE receptor expression on effector cells that may be involved in the initiation or inhibition of IgE-dependent allergic inflammation). Although initially appearing to be somewhat disappointing, these observations do nevertheless contribute to improving our understanding of immunotherapy. Of course changes in many of the parameters assessed have been observed during active immunotherapy in many studies and do

seem to some extent to correlate with clinical benefit in these (as described above and in the introductory section of Chapter 5). However this particular study was fairly unique as it evaluated subjects in whom active immunotherapy had ceased and found, for example, that changes in allergen specific serum IgE and IgG did not seem as important for sustained benefit as they may appear to be during initiation of IT.

Of course blood derived IgE receptor bearing cells could still play a significant role in some of the processes involved in maintaining the efficacy of IT. For instance, were the study to be repeated today, it would be instructive to investigate functional changes in IgE / IgE-receptor allergen trapping mechanisms (e.g. changes in the proportion of receptor bound allergen specific IgE) and indeed effector actions further downstream which may be relevant (e.g. an *in vitro* comparative study of allergen induced lymphocyte proliferation with monocytes derived from subjects who had either continued or discontinued their IT). One further caveat to consider when assessing the effects of prolonged IT is that it is possible that changes in the local microenvironment at sites of allergic inflammation (e.g. the nasal mucosa) that may be relevant to its sustained efficacy may not necessarily be accurately reflected in peripheral blood.

Taken as a whole however, perhaps the most insightful analysis of the outcomes of this study may be that all of them would be compatible with the hypothesis that the mechanism most relevant to the clinical benefit of prolonged IT persisting even after it has been discontinued may be the modification on T-lymphocyte responses to natural allergen exposure (Durham & Till 1998), either by causing an enduring shift in the balance of predominant allergen-specific responses from T_H2 toT_H1 (Till 1997) or by upregulating IL-10 producing adaptive regulatory T cells that are capable of suppressing T_H2 type responses (van Oosterhout & Bloksma 2005).

In fact, although IL-10 secretion was not specifically assessed in our particular study, in a subsequent study increased allergen induced IL-10 secretion from PBMC has been observed in subjects who had received 2 years of grass pollen IT (Nouri-Aria et al. 2004). This observation has been repeated in a very recent study, in which it was noted that increased IL-10 production was observed within a few weeks of the initiation of allergen specific IT and coincided in time with the inhibition of cutaneous

late phase reactions (Francis et al. 2008). In this respect, probably the most relevant observations from the subjects involved in the study described in Section 5 were that not only did both groups (ITA and ITP) demonstrate persistent inhibition of cutaneous late phase responses to *Phleum pratense*, but also had significantly reduced numbers of CD3+ T-lymphocytes and cells containing mRNA for IL-4 at the sites of their intra-dermal allergen challenges (Durham et al. 1999). These findings, indicating a profound and sustained suppression of T_H2 responses associated with prolonged IT, were therefore certainly consistent with the above hypothesis.

Although too vast a topic to discuss in any great depth in this thesis, the significant advances that have taken place over the last few years in beginning to understand the some of the complex mechanisms of how dendritic cells (many of which in atopic individuals express FcεRI of course, as described in Section 6.2.2) promote regulatory T cell response are considered increasingly relevant to the efficacy of allergen specific IT (Larche et al. 2006;Akdis et al. 2006). Therefore a fairly brief synopsis of the most significant points is provided below.

6.6 Regulatory T-cells and Dendritic Cells: a synopsis of recent advances in understanding the roles of these cells in controlling immune responses

Until very recently DCs were thought of mainly as proficient antigen presenters – somewhat akin to highly efficient 'factory ships' that detect and capture antigens then process, package and present them to lymphocytes. However it is now evident that DCs play at least an equal role to T-lymphocytes in deciding the nature of antigen specific responses. Indeed a more appropriate metaphor is that if lymphocytes are the 'generals' that prosecute battles against pathogens, DCs are 'strategic intelligence' assessing potential pathogens and counselling T-lymphocytes whether to tolerate them or, if a specific immune response is required, helping decide the nature of this response. Some recent advances in our knowledge of complex interactions between DCs and T-cells involved in these regulatory decision making processes are summarised in the following sections.

6.6.1 Regulatory T-cells

The roles of different subsets of CD4+ T-cells in immunity are well established. In addition, the existence of putative suppressor T-lymphocytes that could limit harmful immune responses had been suspected for years but identification of such cells proved to be very difficult. This continued to be the case until a series of experiments in the mid-1990s demonstrated that the adoptive transfer of CD25+ depleted CD4+ T-cells into athymic nude mice resulted in multi-organ autoimmune diseases – which could however be prevented if reconstituted CD4+CD25+ cells were also inoculated shortly afterwards (Sakaguchi et al. 1995). CD25, the α-chain of the receptor for interleukin-2, had until then thought to be expressed on activated effector T-cells but this was probably the first indication that some CD4+CD25+ could also be involved in down-regulating immune responses. In the ensuing years a substantive body of evidence has accumulated confirming the presence of at least two distinct subsets of CD4+ T-cells in humans (as well as mouse models) that can inhibit or suppress T-cell activation and consequent harmful immunopathological responses to self or foreign antigens (Shevach 2000;Shevach 2002;Maloy & Powrie 2001;O'Garra & Vieira 2004). These inhibitory T-cells are termed regulatory T-cells (Treg cells).

A major impediment to the study and characterisation of Treg cells in humans has been the lack of a unique or specific cell surface marker allowing them to be identified easily (Romagnani 2006). Nevertheless it is now generally accepted that there are at least two main categories of Treg cells – natural Treg (nTreg) and adaptive Treg (aTreg) cells – that appear to be functionally and phenotypically distinct and develop at different anatomical sites (Bluestone & Abbas 2003).

Natural Treg cells are predominantly generated in the thymus, following which they move on to blood and peripheral lymphoid tissues. These cells are present in all normal individuals where they are thought to represent between 5-10% of the CD4+ lymphocyte population (van Oosterhout & Bloksma 2005;O'Garra & Vieira 2004) and are mainly associated with the induction of tolerance to self antigens. Characteristically they express constitutively high levels of CD25 as well as the co-inhibitory molecule (Chikuma & Bluestone 2003) CTLA-4 (cytototoxic T-

lymphocyte antigen 4) and GITR (glucocorticoid induced tumour necrosis factor receptor), a member of the TNF superfamily (Bluestone & Abbas 2003). However, as has already been discussed, none of these markers are exclusive to nTeg cells.

Perhaps the one relatively exclusive marker for nTreg cells is the transcription factor Foxp3 (Hori et al. 2003;O'Garra & Vieira 2003). The presence of Foxp3 seems to be essential for nTreg cell development (Fontenot et al. 2003) and mutations in the gene encoding for this transcription factor can result in immune dysregulation syndromes such a the X-linked autoimmune and allergic dysregulation syndrome as well as X-linked neonatal diabetes, enteropathy and endocrinopathy syndrome (Romagnani 2006). Until very recently therefore it was thought that the expression of Foxp3 was an essential early step for naive T-cells to become committed nTreg cells in the thymus – however, as all too often proved to be the case in the rapidly progressing study of regulatory mechanisms, the situation has proved to be far more complex than initially thought. For instance, it is possible that under certain circumstances Foxp3 expressing regulatory T-cells could be generated from conventional CD4+ T-cells in the periphery – as several recent studies have reported that CD4+CD25– cells can upregulate Foxp3 after *in vitro* activation (Walker et al. 2003;Chen et al. 2003;Cong et al. 2005;Razmara et al. 2008).

In contrast to nTreg cells, adaptive Treg cells are generated in peripheral lymphoid tissue mainly in response to foreign antigens presented by tolerogenic dendritic cells (which are discussed in greater detail in Section 6.6.4) to T-cells (Bluestone & Abbas 2003;Hawrylowicz 2005). It is now generally accepted that there are at least two major subtypes of aTreg cells – termed T_R1 and T_H3 cells (Hawrylowicz 2005;Romagnani 2006). Although both subtypes do express CD25, its expression is variable and does not appear to be constitutive as in nTreg cells. T_R1 cells do not appear to express Foxp3 and this is also probably the case for the majority of T_H3 cells (Romagnani 2006). It is also now generally accepted that the immunoregulatory actions of both aTreg subtypes are mediated by the secretion of specific inhibitory cytokines – T_R1 cells produce IL-10 and T_H3 cells secrete TGF-β (Bluestone & Abbas 2003;Romagnani 2006).

6.6.2 Interleukin-10 (IL-10)

Although it was originally described as a T_H1 inhibitory cytokine produced by T_H2 cells (Fiorentino et al. 1989;Fiorentino et al. 1991)}, it very soon became apparent that IL-10 is also secreted by T_H1 cells (Del Prete et al. 1993) as well as other cell types – particularly antigen presenting cells like monocytes (de Waal Malefyt et al. 1991a;Parry et al. 1997) and dendritic cells (Akbari et al. 2001;Weigt et al. 2003;Qi et al. 2003) in certain circumstances. IL-10 has several immunosuppressive actions (Moore et al. 2001;Pestka et al. 2004), including many which may have relevance to allergen specific immunotherapy (Jutel et al. 2006). These include:

- It inhibits proliferation of CD4+ T-lymphocytes by its actions on APCs (Fiorentino et al. 1991);
- It potently inhibits secretion of pro-inflammatory cytokines such as IL-1, IL-6, IL-12 (a particularly important cytokine for the generation of T_H1 cells), GM-CSF, TNF-α and, in an autocrine manner, IL-10 itself, from cells of monocyte/macrophage lineage. Furthermore it also suppresses secretion of both CC and CXC chemokines from activated monocytes (Moore et al. 2001);
- It inhibits both the maturation of DCs from monocyte precursors (Buelens et al. 1997) and their expression of MHC class II molecules and co-stimulatory molecules (de Waal Malefyt et al. 1991b;Moore, de Waal Malefyt et al. 2001). As will be discussed shortly, these may be important actions in the generation of aTreg cells – including T_R1 cells, which as described above, further produce IL-10;
- It can also downregulate T_H2 responses (Grunig et al. 1997). Particularly relevant to immunotherapy is the observation that IL-10 can induce PBMCs to differentially produce IgG4 in preference to IgE *in vitro* (Jeannin et al. 1998);
- It has been shown to inhibit IgE induced mast cell activation (Royer et al. 2001) and also cytokine production from mast cells (Arock et al. 1996);
- IL-10 has been observed to reduce eosinophil survival, activation and cytokine production (Takanaski et al. 1994).

6.6.3 Dendritic cells – arbitrators of tolerance or immunity

At almost the same time that the existence of two main Treg subsets was proposed (Bluestone & Abbas 2003), it became apparent that DCs also play a fundamental role in deciding whether the response to a pathogen should be tolerance or immunity (Buckland 2003).

It was already known that DCs can help skew immune response toward a predominantly T_H1 or T_H2 pathway. For instance, DCs of myeloid lineage, producing the archetypical T_H1 stimulating cytokine IL-12, had been shown to be efficient promoters T_H1 cell development from naive CD4+ T-cells (Macatonia et al. 1995) whereas pDCs could be shown to induce T_H2 type responses (Iwasaki & Kelsall 1999). Indeed mDCs and pDCs were at one stage designated DC1 and DC2 cells, based on their ability to promote T_H1 or T_H2 responses respectively (Kalinski et al. 1999;Kapsenberg & Kalinski 1999;Kapsenberg et al. 1999;Moser & Murphy 2000). However it very soon became apparent that the promotion of T_H1 or T_H2 type immunity is not an exclusive property of one or other of these two DC subsets (Schuurhuis et al. 2006). Furthermore it also became apparent that whilst DCs are efficient at promoting adaptive immune responses, they can be equally adept at promoting tolerance through induction of pathogen specific Treg cells (Abbas & Sharpe 2005). Rapidly increasing knowledge of the sequence of events involved between pathogen recognition and capture by DCs and antigen presentation by them to naive T-cells has resulted in significantly better understanding of how and why DCs might promote either immunogenic or tolerogenic pathogen specific T-cell responses (Schuurhuis et al 2006).

6.6.4 Dendritic cells and the generation of adaptive regulatory T-cells

It was soon realised that the classification of DCs into DC1 and DC2 subsets was far too simplistic – in fact the DCs network is a very sophisticated one and DC subsets appear to demonstrate great functional flexibility to induce different T-cell responses taking into account factors such as the nature of the pathogen, the tissue type and microenvironment in which it is encountered, the dose of pathogen, the pattern recognition receptor used to detect it etc before ‘advising’ T-cells to adopt specific

immune or regulatory response phenotypes (Kapsenberg 2003). Some of the important factors that effect this decision making process include:

a. ***Tissue specific DC phenotype***

As discussed in Section 6.2.2, DCs at different sites appear to have different phenotypes, even though broadly following into the categories of mDCs and pDCs (Novak et al. 2004). For instance the majority of oral and nasal DCs have a myeloid lineage but differ in expression of FcεRI (expressed constitutively in both but with higher expression in oral DCs), co-stimulatory molecules (CD40 and CD80/CD86 higher in nasal DCs) and MHC class II expression (higher in oral mDCs) (Allam et al. 2006). In turn, both oral and nasal DCs differ from classical skin Langerhans cells as well as IDEC (inflammatory dendritic epidermal cells), a type of mDC found specifically in inflamed skin in subjects with atopic dermatitis, both of can be can be induced to express FcεRI in atopics and at higher levels than in oral or nasal mucosa (Bieber 2007). Interestingly oral mucosa DCs seem to be quite tolerogenic, and have a propensity to produce the suppressive cytokines IL-10 and TGF-β (Novak et al. 2004;Bieber 2007). In contrast the skin DCs are more likely to be pro-inflammatory – with classical LCs inducing T_H2 mediated inflammation and IDECs promoting predominantly T_H1 responses (Novak & Bieber 2005).

b. ***DC maturity***

The majority of DCs in skin and mucosal tissues are of course relatively immature. Immature DCs are highly efficient at recognising, capturing and internalising antigens (by phagocytosis or receptor mediated endocytosis) and processing them by proteolysis into peptide fragments that can eventually be presented to T-cells. Therefore immature DCs highly express pattern recognition receptors such as Toll like receptors (TLR), immunoglobulin Fc receptors (including FcγR and FcεRI) and C-type lectin receptors. However they have low expression of the co-stimulatory molecules (such as CD40, CD80 and CD86) and adhesion molecules. Although they express high intracellular levels of MHC class II molecules (residing in specialised endocytic compartments called MHC II enriched compartments or MIIC), surface MHC II expression is relatively low when compared to mature DCs (Schuurhuis et al. 2006).

If the conditions are right (particularly the absence of suppressive cytokines like IL-10) DCs rapidly mature and migrate to local lymphoid organs where they can present the processed peptides to naive T-cells by MHC II molecules-T cell receptor/CD3 complex interaction. The process of DC maturation involves a major metamorphosis into non-endocytic antigen presenting cells with changes in cell shape (development of numerous processes called dendrites), loss of the endocytic and phagocytic receptors, upregulation of adhesion molecules and chemokine receptors (e.g. CCR7) that facilitate the migration and greatly increased surface expression of MHC II and co-stimulatory molecules (including the three mentioned above) (Schuurhuis et al. 2006).

In general, it appears that mature DCs are efficient at initiating immunogenic T-cell responses whilst immature DCs promote tolerance (Abbas & Sharpe 2005). This was elegantly demonstrated in an experiment in which immature or mature human monocyte derived DCs were used to repetitively stimulate allogeneic naive CD4+ T-cells. The T-cells stimulated by mature DCs differentiated into activated T_H1 type cells whereas those repetitively stimulated by the immature DCs developed a Treg phenotype (expressing CD25 and CTLA-4 and secreting IL-10) (Jonuleit et al. 2000). Intriguingly this *in vitro* mechanistic study suggested two separate ways DCs could significantly contribute to the efficacy of allergen specific immunotherapy *in vivo,* by promoting either immune deviation or tolerance (or possibly both in the long term).

c. ***Receptors involved in pathogen recognition and capture***

Dendritic cells express a large variety of receptors that allow them to recognise, capture and internalise pathogens. Some, particularly receptors for the Fc domain of immunoglobulins, confer a degree of antigen specificity. The functional consequences of 'antigen focusing' by FcεRI on DCs, in particular, have already been discussed in this thesis.

However DCs, unlike T-lymphocytes for instance, are not pathogen specific cells – indeed they possess the vital ability to detect danger patterns on any number of pathogens and instruct the adaptive immune system on appropriate responses. DCs

possess a variety of pattern recognition receptors such as lectin, mannose and heat shock protein receptors but ones that have attracted most recent attention are the ten TLR (Toll like receptors). TLR of course mediate innate immunity by recognising conserved microbial constituents (Beutler 2002) such as lipoproteins (TLR2-TLR1), peptidoglycans and zymosan (TLR2-TLR6), lipopolysaccharides, LPS (TLR4), viral dsRNA (TLR3) and bacterial DNA containing CpG motifs (TLR9 found on pDCs) (Schuurhuis et al 2006) but intracellular signals that result from the activation of at least some of them can also modify adaptive immune response (Abreu & Arditi 2004). For instance, *in vitro* stimulation of mDCs by three different TLR activators LPS, peptidoglycan or zymosan induced different levels of inhibitory (IL-10) or pro-inflammatory (IL-12) cytokines and resulted in generation of different T-cell phenotypes (Qi et al. 2003). Similarly, activation of oral mucosal Langerhans cells by a TLR4 agonist resulted in CD4+ T-cells showing upregulation of co-inhibitory molecules (B7-H1 and B7-H3), downregulation of the co-stimulatory molecule CD86 and upregulation of Foxp3 and the inhibitory cytokines IL-10 and TGF-β1 as well as the T_H1 cytokine IFN-γ (Allam et al. 2008).

The facility of these receptors of innate immunity on DCs to alter antigen specific T-cell responses may be relevant to the allergic condition – for instance, in a very recent study it was observed that circulating pDCs from atopic individuals stimulated with the TLR9 specific ligand, CpG-DNA, have a greatly reduced propensity to produce IFN-γ (the prototype T_H1 cytokine) than those from non-atopic subjects despite the numbers of pDCs and TLR9 expression being similar in the two groups (Tversky et al. 2008).

d. ***The role of co-stimulatory or co-inhibitory molecules***

The antigen presenting interaction between MHC II molecules on APCs and T-cell TCR-CD3 complexes needs an additional non-antigen specific co-stimulatory second signal for activation to occur. This involves interaction between accessory signalling molecules on the presenting DC and the T-cell receiving the antigen. Recognised pairs of co-stimulatory molecules include CD40 and CD40 ligand, CD80 or CD86 and CD28 on APCs and T-lymphocytes respectively. CD80 and CD86 on APCs can also interact with the co-inhibitory molecule CTLA-4 on T-cells and in fact have a

much higher affinity for this molecule than for CD28 (Abbas et al. 1996). It is generally thought that antigen presentation to T-cells without concomitant co-stimulation (as is likely with immature DCs with their low levels of expression of co-stimulatory molecules) results in T-cell anergy whereas efficient co-stimulation by mature DCs leads to effector response (Schwartz 2003). Recently it has been postulated that such a mechanism may cause temporary functional unresponsiveness but not necessarily anergy. Instead it has been postulated that, for instance, the relatively few numbers of CD80 or CD86 molecules expressed on immature DCs may still effectively bind co-inhibitory CTLA-4 molecules and, in the presence of inhibitory cytokines such as IL-10 and TGF-β promote regulatory rather than effector T-cells (Abbas & Sharpe 2005). Although this still remains an attractive but unproven hypothesis, there seems little doubt that accessory signalling molecules are relevant to antigen specific tolerance inducing interactions between DCs and T-lymphocytes.

e. ***Tissue factors***

Whilst the contact interaction between DCs and T-cells involving ligation of TCRs by MHC class II molecules together with the engagement of the accessory signalling molecules (signals 1 and 2 respectively) is important in determining whether the T-cells mount an effector response or not, the decisive factor as to the precise nature of the response (signal 3) is thought to be the local microenviroment where DCs encounter the pathogen (Kalinski et al. 1999). The presence or absence of many factors such as pro-inflammatory cytokines (e.g.IL-12 family members, IFN-γ, IL-10, TGF-β, MCP-1), eicosanoids, heat shock proteins, extracellular matrix components etc can significantly alter DC activation and maturation and thus effect the nature of the DC modulated adaptive reponse to a particular antigen (Kapsenberg 2003).

6.6.5 Dendritic cells and immunotherapy: a brief summary of recent advances

Better understanding of the regulatory function of DCs and how all of the factors described above may effect this (Novak & Bieber 2008) has stimulated research on how to utilise DCs to enhance immunotherapy for conditions as diverse as cancers, autoimmune diseases, transplant medicine and, of course, allergen specific immunotherapy (Figdor et al. 2004). The basic concept is that antigens, or allergens,

should be targeted to one or more specific type of receptors on DCs at particular anatomical sites to achieve the desired T-cell response (Tacken et al. 2007).

In allergen specific immunotherapy the simplest application of this idea has been to try and exploit the constitutively high level of FcεRI expression of DCs in the oral mucosa and, as discussed above, their essentially tolerogenic nature through sublingual allergen specific immunotherapy or SLIT (Akdis et al. 2006). One example of the success of this approach was demonstrated in a recent study in which patients who responded to birch pollen SLIT, an increased number of circulating T_R1 cells with increased IL-10 but reduced IL-4 and IFN-γ expression was noted within four weeks of commencing SLIT. However after one year of SLIT, IL-10 secreting Treg cells were reduced but there was now evidence of allergen specific immune deviation to a predominantly T_H1 response (increased IFN-γ but little IL-4 production) (Bohle et al. 2007).

A slightly more sophisticated approach involves conjugating the allergen to DC receptor ligands – particularly TLR stimulatory sequences. The theory is that if a particular type of TLR is stimulated at the same time as allergen is presented to a DC via FcεRI bound IgE, the TLR ligand will act as an adjuvant and modulate the subsequent allergen specific T-cell response. Such an approach has been recently been trialled using Amb a 1 (the ragweed pollen antigen) conjugated to a synthetic DNA sequence containing CpG motifs that is a TLR9 specific ligand (Marshall et al. 2001;Tulic et al. 2004;Simons et al. 2004;Creticos et al. 2006). Two recent clinical studies of SCIT using this conjugate in ragweed sensitive rhinitics did demonstrate clinical efficacy and, as might have been anticipated, evidence of immune T_H1 deviation (Tulic et al. 2004;Creticos et al. 2006). This ability of this allergen conjugate to skew allergen specific cytokine responses from T_H2 towards T_H1 *in vivo* was also demonstrated in another study involving ragweed allergic patients receiving a short subcutaneous course of the conjugate (Simons et al. 2004). A grass pollen and monophosphoryl lipid A conjugate, this time simultaneously targeting TLR4 and FcεRI, has also shown similar promise (Mothes et al. 2003;Drachenberg et al. 2001)

The development of immunotherapy strategies targeting DCs is a particularly fertile area of research, especially in cancer medicine (although the idea there is more to 'switch on' immunity rather than tolerance) (Tacken et al. 2007). However there is little doubt that these DC based vaccine strategies will also eventually be used in allergy. One approach that could, for instance, be effective would be to combine allergen specific IT with strategies aimed at modulating co-stimulatory or co-inhibitory molecules – such as a novel fusion protein, CTLA-4.Ig or abatacept, which is already available and has been shown, when combined with MHC II-TCR ligation *in vitro*, to convert naive CD4+ T-cells to CD4+CD25+ Treg cells (Razmara et al. 2008). Thus it appears likely that the presence of FcεRI on DCs coupled with increasing knowledge of how to accurately and reliably manipulate DC functions will result in greater efficacy and safety of allergen specific immunotherapy in future.

6.7 Conclusion

Four decades have now passed since the discovery of IgE but it is only in the last ten to fifteen years that rapid and significant advances have been made in our knowledge of FcεRI – the high affinity receptor for IgE. Fascinating insights have been gained not just into the structure of this receptor but also how the peculiar topology of the IgE binding site on the α-chain of FcεRI is fundamental to the extremely potent stability of the binding of IgE to this receptor. Our knowledge of events both in the cell membrane and intracellular signalling pathways has also advanced apace.

However perhaps one of the most exciting developments, particularly with regard to our understanding of the cellular basis of IgE mediated allergic inflammation, has been the identification of a greatly increased repertoire of cells, both in blood and in tissue, that possess functional FcεRI on their surfaces. These now include several types of antigen presenting cells (APCs), especially those of monocytes/macrophage lineage such as mDCs, on whom the surface expression, particularly in atopic subjects, appears to be inducible. As discussed above, the presence of strongly bound allergen specific IgE on the FcεRI on these cells, may greatly enhance the ability of these cells to present allergen to T-lymphocytes (cells that are now considered to be particularly important pro-inflammatory and regulatory cells in allergic

inflammation). Furthermore, the advances in knowledge mean that even the cell types that have long been known to constitutively express large numbers of surface FcεRI, mast cells and basophils, are now considered to have broader roles in allergic inflammation than just being fundamental in Type I IgE mediated hypersensitivity reactions. Mast cells and basophils, as well as FcεRI bearing APCs, are now known to be able to synthesise, store and secrete a variety of cytokines that have important roles in the initiation and maintenance of allergic type inflammatory reactions. The advances in knowledge of the IgE – FcεRI interaction as well as of the cells that express FcεRI are leading to the development of novel therapeutic agents (such as omalizumab). However even when trying to elucidate the mechanisms of more conventional therapeutic agents, such as cyclosporin A, or potentially disease modifying therapies such as allergen specific immunotherapy, due consideration should be given not just to the possible effects of these therapies on FcεRI bearing cells but also to how the efficacy of the therapy may be enhanced through functional manipulation of particular FcεRI+ cells (such as dendritic cells).

The four studies forming the core of this thesis contributed, at least to a small extent, to some of these advances in understanding the nature and roles of FcεRI bearing cells. In the introduction (Chapter 1) I attempted to summarise the diverse studies that had taken forward our knowledge of IgE and its high affinity receptor in the first three decades after the discovery of IgE and how these related to the studies described in these ‘core papers’. In this discussion chapter I have tried to show how the observations in these studies have been taken forward, some by researchers in he same department where the research for these papers was originally undertaken, but many by researchers in other institutions. Whilst research in all these areas still continues, this thesis has tried to summarise an exciting epoch in research into a fascinating immune receptor.

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