APPROACHES FOR THE MODULATION OF ALLERGEN-SPECIFIC TH2 IMMUNITY

GILLIAN HALL

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Edinburgh University Medical School

Abstract

The prevalence of allergic diseases, such as asthma, rhinitis, eczema and food allergies has increased dramatically over the last few decades and is now a major health and economic burden throughout the developed and developing worlds. Type I (immediate) hypersensitivity reactions are mediated by immunoglobulin E (IgE) responses directed against innocuous environmental antigens, such as pollen, house-dust mites or animal dander. It is the resulting release of pharmacological mediators by IgE-sensitised mast cells that cause the symptoms of asthma and allergic rhinitis. The induction of IgE is dependent on $CD4^+$ T cells of the Th2 phenotype which are characterised by the production of specific cytokines (IL-4, IL-5 and IL-13). In contrast, the presence of allergen reactive Th1 cells, which secrete IFN-γ, in non-allergic healthy individuals suggests that Th1 immunity is not damaging to the host and is possibly associated with protective immunity.

It is clear with the high incidence of atopic disorders combined with existing treatments, which are in general symptomatic, that there is a requirement for new therapeutic agents. Since $CD4^+$ T cells play an important role in the response to allergens they are an obvious target for drug development and they can be targeted directly, with the aim of inducing specific tolerance. A second strategy for inhibiting the synthesis of Th2 cytokines may be achieved by promoting the induction of Th1 immunity.

Therefore, the main aim of this study was to investigate these different approaches for the modulation of Th2 immunity to the major house dust mite allergen Der p 1. Tolerance induction or the promotion of Th1 responses were attempted by intranasal delivery of antigen alone, by the systemic or mucosal delivery of Der p 1 in PLG polymer microparticles (MEA) and finally, by intranasal administration with chitosan, an enhancer of epithelial permeability. In order to investigate the efficacy of the regimens of vaccination, an adjuvant free model of Th2 cytokine-mediated allergic inflammation was developed *in vivo* in $H\text{-}2^b$ mice.

Vaccination with microencapsulated antigen failed to elicit a Th1 response or induce tolerance despite altering the kinetics, dose and method of delivery. In fact, the Th2 phenotype was usually exacerbated following administration of

MEA/Der p 1 particles. Intranasal co-administration of antigen with chitosan inhibited Th2 cytokine production but not as a result of the tolerance induction. Similarly, high doses of soluble peptide delivered intranasally, failed to tolerise allergen-specific Th2 immunity.

In conclusion, the redirection of the Th2 immune response and the induction of tolerance were difficult to achieve. However, chitosan which was not as extensively researched as the other approaches may prove to be of therapeutic value.

Declaration

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Gillian Hall

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Dedication

For Mam, Dad and Allison.

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Abbreviations

Ab	Antibody
AHR	Airway Hyperresposiveness
APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
BAL	Broncho-Alveolar Lavage
BCG	Bacillus Calmette Guérin
CFA	Complete Freunds' Adjuvant
CLIP	Class II-Associated Invariant Chain Peptide
CLN	Cervical Lymph Nodes
CPD	Chronic Pulmonary Disease
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
Der p 1	*Dermatophagoides pteronysinnus* allergen group 1
DT	Diphtheria Toxin
DTH	Delayed Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme Linked ImmunoSorbant Assay
ENV	Environmental
EPO	Eosinophil Peroxidase
ER	Endoplasmic Reticulum
FAE	Follicle-Associated Epithelium
GEN	Genetic
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HAI	Haemagglutinin Inhibition
HLA	Human Leukocyte Antigen
ICAM	Intercellular Adhesion Molecule
ICOS	Inducible Costimulator
IEL	Intraepithelial Lymphocyte
IFA	Incomplete Freunds' Adjuvant
IFN	Interferon
Ig	Immunoglobulin

Ii	Invariant Chain
IL	Interleukin
IP	Intra-peritoneal
iTAC	Interferon T Cell-α Chemoattractant
IV	Intra-venous
KLH	Keyhole Limpet Haemocyanin
LAG	Lymphocyte Activation Gene
LCMV	Lymphocytic Choriomeningitis Virus
LPL	Lamina Propria Lymphocyte
MALT	Mucosa-Associated Lymphoid Tissue
MBP	Myelin Basic Protein
MEA	Microencapsulated Antigen
MC	Mast Cell
MLN	Mesenteric Lymph Nodes
MiG	Monokine induced by Interferon γ
MHC	Major Histocompatibility Complex
NALT	Nasal Associated Lymphoid Tissue
NK	Natural Killer
OVA	Ovalbumin
PAF	Platelet Activating Factor
PALN	Para-Aortic Lymph Node
PAM	Pulmonary Alveolar Macrophages
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PLG	Poly Lactide co-Glycolide
PLL	Poly L-Lysine
PLP	Proteolipid Protein
RNA	Ribo-Nucleic Acid
RIP	Rat Insulin Promoter
SC	Subcutaneous
SI	Stimulation Index
TAP	Transporter Associated with Antigen Processing

TCR	T Cell Receptor
Th	T helper
Th0	T helper 0
Th1	T helper 1
Th2	T helper 2
TGF	Transforming Growth Factor
TLN	Tracheal Lymph Node
TNF	Tumour Necrosis Factor
VCAM	Vascular Cell Adhesion Molecule

Chapter 1

General Introduction

1.1 T Cell Subsets

There are two distinct T cell lineages - α:β and γ:δ. In mice and humans most T cells (about 95-99%) express α:β T cell receptors, whereas the minority (about 1-5%) express γ:δ T cell receptors. In sheep the ratio of α:β to γ:δ T cells is about 50:50. The $\alpha:\beta^+$ T cells can be further distinguished by the presence of a surface co-receptor protein, either CD4 or CD8. In general, $CD8^+$ T lymphocytes recognise peptide fragments (epitopes) derived from endogenous proteins (i.e. proteins that are synthesised within the cell or artificially introduced directly into the cytoplasm or the nucleus). These are presented on the cell surface in association with MHC class I molecules. $CD4^+$ T lymphocytes recognise peptide epitopes derived from exogenous or membrane proteins, which enter the cell via endocytosis or phagocytosis and are primarily, degraded by lysosomal proteases. These are presented on the cell surface bound to MHC class II molecules.

1.1.1 MHC Class I Molecules

MHC class I molecules consist of a heavy chain, β_2-microglobulin and a peptide. The heavy chain is 45 kDa and is encoded by the genes HLA-A, HLA-B and HLA-C on chromosome 6 in humans and chromosome 17 in mice (H-2K, H-2D, H-2L). The gene for β_2-microglobulin is located on chromosome 15 in humans and on chromosome 2 in mice. The peptide is usually eight to ten amino acids long and is derived from cellular proteins or foreign antigens [1]. The MHC genes have extensive polymorphism, which is vital for antigen presentation and recognition by T cells. The two extracellular domains α_1 and α_2, form the peptide-binding groove and it is here where most of the polymorphic residues are located. Anchor residues determine peptide binding to MHC molecules. The side chains of the residues are buried in pockets within the MHC peptide-binding groove, usually one anchor

residue is located at the carboxyl terminus and a second at a varying internal position depending on the identity of the class I allele [2].

Cytoplasmic antigens are degraded into short peptide fragments by proteasomes. These are large cylindrical complexes with a hollow core consisting of the active sites of proteolytic subunits. Certain features of proteasomes distinguish them from most other enzymes. First, proteasomes are multicatalytic, i.e. they contain independent active sites and may possess as many as five to nine different activities [3]. Secondly, they degrade proteins in a processive fashion, i.e. one molecule is degraded to completion before another is processed [4].

The delivery of peptides from the cytoplasm into the endoplasmic reticulum is facilitated by TAP (transporter associated with antigen processing). TAP is composed of two homologous integral membrane proteins, TAP1 and TAP2, that act in an ATP-dependent manner. ATP hydrolysis is required for the TAP1/2 complex to translocate peptides into the ER but is not required for peptide binding [5].

In the endoplasmic reticulum, newly synthesised MHC class I α chains bind to calnexin, a chaperone protein which functions to retain the MHC class I molecule in a partially folded state. B_2-microglobulin then binds to the α chain, resulting in the dissociation of the complex from calnexin and the subsequent association with a second chaperone protein, calreticulin. Tapasin, a TAP1-associated protein, forms a bridge between the MHC class I molecules and the TAP complex, until the delivery of a suitable peptide from the cytosol. Finally, peptide binding to the partially folded heterodimer enables its release from the TAP/tapasin/calreticulin complex, allowing the now fully folded MHC class I molecule to leave the ER and be transported to the cell surface [6].

1.1.2 MHC Class II Molecules

In humans, class II molecules are encoded by the polymorphic HLA-DR, HLA-DQ and HLA-DP genes and expressed as two noncovalent heterodimers of two transmembrane polypeptides, the α-chain (35 kDa) and the β-chain (27 kDa) [7]. MHC class II molecules mainly present peptides derived from internalised proteins that have entered the cell by endocytosis. This process leads to the containment of the protein antigen in endosomes, which become increasingly acidic, as they progress

deeper into the cell. The endosomal proteins are eventually degraded to peptides by the action of acid proteases.

In the endoplasmic reticulum, MHC class II-associated invariant chain (Ii) assembles with newly synthesised MHC class II molecules. This prevents premature binding to peptides transported into the ER lumen by the TAP transporter, or to endogenous peptides newly synthesised by the cell itself. Ii chain binds to the MHC class II molecule with part of its polypeptide chain lying within the peptide-binding groove thus preventing binding of other peptides. The invariant chain also targets the delivery of MHC class II molecules from the ER to an appropriate acidic endosomal compartment, where peptide loading can occur. Over a period of 2-4 hours in this compartment, the invariant chain is cleaved by proteases such as cathepsin S or cathepsin L [8]. Initial cleavage generates a truncated form of the invariant chain that remains bound to the MHC class II molecule and is retained within the endocytic compartment. Subsequent cleavage releases the MHC class II molecule from the membrane-associated fragment of Ii, leaving a short fragment of Ii, called CLIP (class II-associated invariant chain peptide) still bound to the MHC class II molecule. The class II-like molecule DM, binds to the MHC/CLIP complex, catalysing the release of CLIP which enables subsequent binding of antigenic peptides, before transportation to the cell surface. The DM molecule also binds to and stabilises empty MHC class II molecules that would otherwise aggregate [9]. Invariant chain-deficient mice ($Ii^{-/-}$) mice have impaired ability to present MHC class II-restricted antigens, and reduced numbers of $CD4^+$ T cells [10]. In contrast, $DM^{-/-}$ mice have normal amounts of class II molecules at the cell surface, but most of these are associated with invariant chain-derived CLIP peptides. These mice contain large numbers of $CD4^+$ T cells, which is indicative of positive selection in the thymus [11].

1.1.3 Presentation of Exogenous Antigens on MHC Class I Molecules

The concept that MHC class I-restricted presentation is only associated with the cytoplasmic degradation of cellular (endogenous) proteins, and is considered inaccessible to exogenous antigens, is not strictly true. Certain exogenous elements can gain entry into this so-called endogenous pathway by a mechanism termed cross-presentation [12, 13, 14 reviews]. Class I-restricted presentation of exogenously derived antigen is particularly important for priming CTL responses, especially to

proteins expressed within extra-lymphoid (peripheral) compartments. This cross-presentation was first implicated in studies examining CTL induction in response to minor histocompatability antigens [15]. Cross-priming has subsequently been shown to occur for viral proteins [16], protein-coated spleen cells [17] and tumour antigens [18]. Bevan proposed that cross-presentation may provide the immune system with a mechanism to detect and respond to tissue-tropic viruses that do not infect APCs [19]. Bennett et al. showed that CTL induction by cross-priming with cell associated ovalbumin required the active involvement of $CD4^+$ helper T cells. Most importantly, the involvement of this $CD4^+$ population was only effective when both the helper and CTL determinants were recognised on the same APC [20]. An earlier study using OVA bound to latex beads, suggested that class II-restricted T cell help was important for CTL induction but did not address the APC requirements [21].

Cross-priming in the context of T cell priming has just been discussed but the cross-presentation of exogenous antigens can also contribute to T cell tolerance, specifically $CD8^+$ T cell deletion. This mechanism is often referred to as cross-tolerance. An early study by von Boehmer and Hafen showed that maturing $CD8^+$ T cells could be tolerised to minor histocompatability antigens expressed on thymic epithelial cells, even when such cells did not carry the restricting MHC class I elements [22]. It was thought that APCs of haematopoietic origin were involved, resulting in thymic cross-tolerance. Peripheral cross-tolerance has also been demonstrated in transgenic rat insulin promoter (RIP)-mOVA mice [23]. OVA-specific $CD8^+$ T cells were adoptively transferred into RIP-mOVA mice which initially resulted in T cell activation (in those lymph nodes draining OVA-expressing tissues) but eventually led to T cell deletion.

This deletion of $CD8^+$ T cells via cross-presentation raised a conceptual paradox. The cross-presentation of peripheral exogenous antigens occurs by professional APCs which usually results in immunogenic rather than tolerogenic T cell responses. This was demonstrated by Ohashi et al. and Oldstone et al. who found that naïve $CD8^+$ T cells ignored lymphocytic choriomeningitis virus (LCMV) proteins expressed exclusively by pancreatic β-cells (i.e. non-professional APCs) but became autoaggressive when the virus was able to access professional APCs [24, 550]. A possible explanation for the existence of some models that show ignorance

of islet antigens whereas others observe tolerance is the effect of antigen dose. Kurts et al. demonstrated that the concentration of self antigen was critical in the induction of ignorance vs tolerance [25]. There was no detectable cross-presentation when low concentrations of ovalbumin were expressed in the pancreatic islets of transgenic mice, with the $CD8^+$ T cell compartment remaining ignorant of OVA. However, in mice expressing higher doses of OVA cross-presentation was detectable resulting in peripheral deletion of OVA-specific $CD8^+$ T cells. This phenomenon was confirmed by reconstituting the bone marrow compartment with cells incapable of presenting OVA, which resulted in a conversion from deletional tolerance to ignorance.

Exogenous antigens can gain access to the MHC class I-restricted pathway by a number of mechanisms. Regurgitation involves antigen degradation by phagocytosis with subsequent release of fragments for binding to cell surface class I [26]. A number of groups have demonstrated that the heat shock protein gp96 can mediate cross-presentation [27, 28]. As yet, the APCs targeted by gp96 have not been identified but the overexpression of certain proteins in transgenic models or viral infection could promote increased expression of this stress-induced protein, thus facilitating class I presentation. Access via the cytosolic pathway is another mechanism. In this case, antigens in the extracellular fluids can be internalised into phagocytes by phagocytosis (large particles) [29, 30], or macropinocytosis (large scale fluid phase uptake involving small particles/soluble antigens). Dendritic cells and macrophages have been implicated in the macropinocytosis mechanism [31, 32].

1.2 $CD4^+$ T Cells

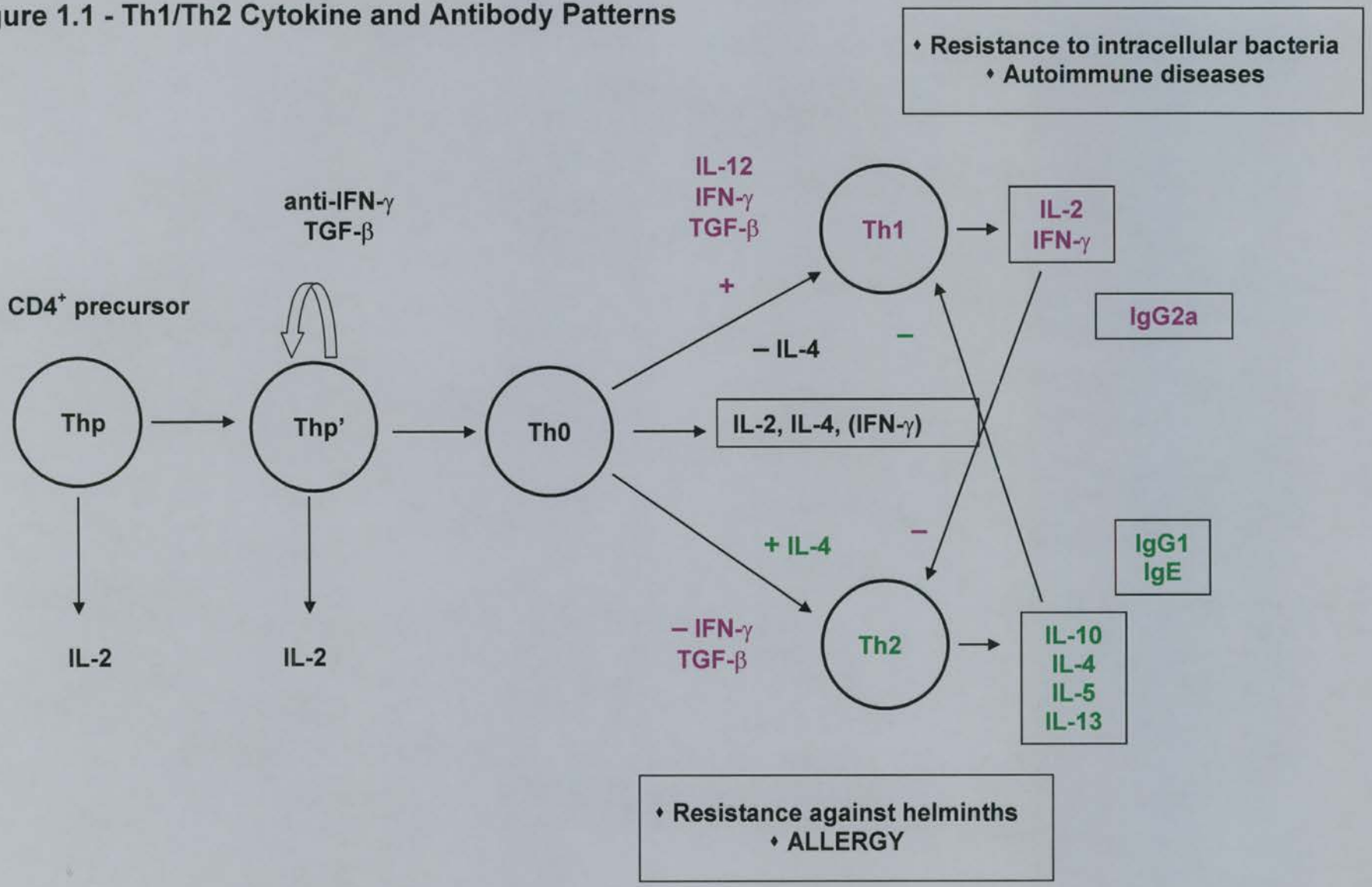
1.2.1 The Th1 /Th2 Paradigm

The T helper 1 (Th1) and Th2 patterns of cytokine production were originally described among mouse $CD4^+$ T cell clones [33, 34] and later among human T cells [35]. Since then, multiple parameters of T cell activation have been discovered that influence Th1 /Th2 development:

- Type of APCs present at site of antigen exposure
- Antigen dose
- Antigen structure, particularly the affinity of the processed peptides for MHC and TCR
- Levels of costimulation (positive or negative signals) during T cell priming
- Genetic background
- Pathogen derived materials
- Presence of cytokines in the priming milieu (see figure 1.1, pg 25).

Usually, Th1 /Th2 priming (directly or indirectly) is influenced by several of the above factors. In addition, there is strong evidence suggesting that Th1 and Th2 cells are not derived from distinct lineages, but develop from the same Th-cell precursor under the influence of environmental and genetic factors acting at the level of antigen presentation. When first stimulated by antigen on APCs, naïve $CD4^+$ T cells initially produce IL-2, and then differentiate into phenotypes that secrete other cytokines. IL-3 has also been implicated in the differentiation and growth of various lymphocyte lines [36]. However, unlike IL-2, IL-3 appears to primarily influence immature lymphocyte differentiation. The environmental and genetic factors that influence Th1 /Th2 differentiation act mainly by determining the predominance of a given cytokine in the microenvironment of the responding Th cell.

Figure 1.1 - Th1/Th2 Cytokine and Antibody Patterns



1.2.2 Contribution of Cells During T Cell Priming

IL-12 and IFN-γ stimulate differentiation into Th1 cells, whereas IL-4 induces Th2 cell differentiation [37]. In a primary response, the frequency of antigen-specific T cells is very low (e.g. 1 in 10^5) so other cell types may supply the appropriate cytokine for differentiation. IL-12 is made by many cell types, but particularly by macrophages in response to certain microbial products [38, 39]. Natural killer (NK) cells synthesise IFN-γ in response either to IL-12 [40] or antigen-mediated cross-linking of antibody bound to Fc receptors [41].

There has been some discussion in the last few years concerning which cell type produces IL-4 and is thus involved in initial Th2 development. Possible candidates include mast cells/basophils, [42] eosinophils [43], the LECAM-1^{dull} subset of $CD4^+$ T cells [44], the $CD4^+NK1.1^+$ T cell subset [45] or naïve T cells themselves [46, 47].

$CD4^+NK1.1^+$ T cells were prime candidates for a while based on the fact that on recognition of the conserved MHC class I-like CD1 molecule, they released bursts of IL-4. However, a later study showed that early IL-4 production in the lymph nodes and development of Th2 responses against L. major infection, originated from a $CD4^+NK1.1^-$ T cell population that was β_2m independent (beta2-microglobulin-deficient mice have been shown to contain strongly reduced numbers of $NK1.1^+$T cells) [48].

1.2.3 Costimulation

Two signals are required for the optimal activation of naïve T cells. One signal is delivered by the engagement of the TCR by Ag-MHC complexes on APCs; the other signal is the ligation of CD28 on T cells by either B7.1 (CD80) or B7.2 (CD86) molecules on APCs [49, 50]. CD28 is expressed by resting and activated T cells. CTLA-4 is expressed on the activation of T cells and is related to CD28. However, CTLA-4 binds the B7 molecules with a higher affinity than CD28 and

studies suggest that it is predominantly a negative regulator of T cell proliferation [50-52].

A number of studies have suggested that CD28 ligation plays an important role in Th cell differentiation [49, 53] and in particular can selectively regulate Th1 and Th2 induction. Thompson et al. hypothesised that the strength of the TCR signal, in conjunction with the strength of costimulatory signals delivered by CD28, can influence the generation of Th1 and Th2 cells. This hypothesis was supported by *in vivo* [47, 54] and *in vitro* [55-58] studies from the Bottomly group. In one of their later studies, they investigated whether the costimulatory requirements of Th1 and Th2 generation *in vitro* might vary with either a strong or weak TCR signal in the presence or absence of B7 costimulatory molecules [58]. In this system, Th2 cells were generated using altered peptide ligands (Ag peptides with a single amino acid substitution at a position known to contact the TCR) or with low doses of wild-type peptide. Th2 cells were dependent on a weak TCR signal and CD28/B7 interactions, as disruption of CD28/B7 interactions inhibited the priming of Th2 cells, and cross-linking CD28 with anti-CD28 antibody augmented the priming of Th2 cells.

Studies in various disease models have proposed that the molecules themselves may play a role in T cell differentiation, with B7.1 favouring Th1 induction and B7.2 favouring Th2 induction [59-61]. In an *in vivo* model of experimental allergic encephalomyelitis, Th1 cells are associated with disease phenotype. Kuchroo et al. demonstrated that anti-B7.1 treatment (resulting in Th2 expansion) ameliorated the disease whereas anti-B7.2 exacerbated the disease [59]. Similar results have been observed in allergic asthma mouse models [61] and also in atopic asthmatic subjects [62].

Larché et al. examined the requirements of CD80 and CD86 on allergen-induced T cell proliferation and cytokine production in peripheral blood and bronchoalveolar lavage from atopic asthmatic subjects. Administration of CTLA-4-Ig fusion protein or anti-CD86 mAb to allergen-stimulated PBMC cultures inhibited proliferation and IL-5 and IL-13 production. Antigen presentation by airway APCs (from BAL) and allergen-induced proliferation from airway T cells was also CD86 dependent. These results supported those of Keane-Myers et al. who found that administration of anti-B7.2 antibody to OVA-treated A/J mice abolished allergen-

induced airway hyperresponsiveness, pulmonary eosinophilia and elevation in serum IgG1 and IgE levels. Lung IL-4 and IL-5 mRNA and bronchoalveolar lavage fluid IL-4 and IL-5 protein levels were also reduced with no significant changes in IFN-γ message or protein levels. This observation concerning IFN-γ was in contrast to the results of Tsuyuki et al. [63]. They observed an increase in IFN-γ levels in *in vitro*-activated lung T cells after anti-B7.2 administration in a similar model of antigen exposure using SV129mice. These differences were thought to be due to mouse strain differences (the A/J strain has an inherent susceptibility to develop Th2 responses, so pathways associated with IFN-γ production may be redundant in these mice) and also in cytokine measurement. In Keane-Myers study, cytokines were measured in BAL fluids taken at the time of measurement of the allergic response, whereas in Tsuyuki's study, IFN-γ levels were derived from isolated lung cells stimulated *in vitro* with anti-CD3 Abs.

In contrast, treatment with anti-B7.1 mAb had no effect on allergen-induced airway hyperresponsiveness, IgE production or cytokine production, however, it significantly suppressed pulmonary eosinophilia. This finding was consistent with that of Harris et al. who demonstrated that blockade of B7.1 with a mutant form of CTLA-4-Ig (which specifically blocked B7.1 signalling) reduced Ag-induced tissue eosinophilia [64].

A study by Mathur et al. compared the effect of *S. mansoni* sensitisation and challenge in wild-type mice, CD28KO mice and mice treated with monoclonal antibodies to CD80 and CD86 [65]. Previous related studies had shown that administration of CTLA-4-Ig after primary immunisation with *S. mansoni* antigens could cause immune deviation from a Th2-like response to a Th1-like response [66]. However, the ability of CTLA-4-Ig to inhibit the interaction of both CD28 and CTLA-4 to both CD80 and CD86 meant that the specific roles of the B7 molecules in the induction of T lymphocyte-mediated asthmatic airway inflammation remained unclear. The study in 1999 by Matur et al. addressed these issues and suggested that CD28, not CTLA-4 was the essential B7 receptor for the induction and progression of Th2-mediated allergic airway inflammation in the *S. mansoni* model. In addition, antibody treatment directed against either CD80 or CD86 alone had a partial and similar inhibitory effect on the development of allergic inflammation. This is in

agreement with Schweitzer and colleagues who reported that CD80 and CD86 costimulatory signals were basically equivalent because both could elicit IL-4 as well as IFN-γ by anti-CD3-stimulated $CD4^+$ T cells [67]. Similarly, *Heligmosomoides polygyrus*-infected mice seem relatively resistant to the effect of treatment with antibodies to either B7 ligands [68]. However, the immune and inflammatory reactions could be blocked when antibodies to both molecules were used together.

These results are in contrast to the previous studies already discussed [61, 63, 64], who all suggest that either CD80 or CD86 is the dominant costimulatory molecule involved in allergic airway inflammation. However, the latter two studies did observe a reduction in eosinophilic recruitment into airways after treatment with only the anti-CD80 antibody.

Most investigators agree that the requirements for costimulatory molecules during immune responses are quite complex and depend on the system studied. There are clear differences in the kinetics of expression and also on which cell type these molecules are expressed. Lung macrophages [64] and lung B cells [63] have been shown to predominantly express B7.2 following inhalational exposure to OVA of mice of two different genetic backgrounds. Mathur et al. demonstrated by reverse transcriptase/PCR and FACs analysis that both CD80 and CD86 were expressed on lung cells from naïve animals and were upregulated within 24 hours of antigen challenge. They also found that both B7 molecules were expressed on eosinophils from the allergic lung with greater expression of CD80 than CD86 on these inflammatory cells.

Although treatment with antibodies to either CD80 or CD86 resulted in a partial reduction in the development of allergic airways in the study by Mathur et al., only anti-CD86 treatment significantly depressed systemic production of IgE [65]. Treatment with anti-CD86 mAbs has previously been shown to result in deficiencies in germinal centre formation and isotype switching [69]. Thus, while local lymphocyte responses to antigen may be driven equally by CD28/CD80 or CD28/CD86 ligation, CD86 seemingly plays the predominant role in systemic B cell response in the mouse.

Recently, another member of this costimulatory family has been identified. Inducible co-stimulator (ICOS) is a homodimeric protein of relative molecular mass

55K-60K exhibiting 20% homology with CD28. However, unlike CD28, ICOS is not present on naïve T cells; instead it is upregulated after T cell activation and retained on many memory T cells. Hutloff et al. proposed that ICOS was involved in the late phase of activation because nearly all $ICOS^+$ T cells expressed CD45RO (a cell surface marker on memory/effector T cells) [70].

In humans, ICOS binds B7h, a molecule expressed by APCs with homology to CD80 and CD86 [71]. Murine homologues of ICOS and B7h (B7RP-1) have also been identified [72]. Data from ICOS-deficient mice have shown that this molecule plays a critical role in T-B cell interactions, is essential for germinal centre formation and humoral immune responses [73, 74]. The inability of ICOS-deficient mice to generate germinal centres is due to their impaired ability to upregulate CD40 ligand [75]. This was overcome by CD40 stimulation, which suggests that ICOS costimulation is mediated, at least in part, via regulation of the CD40-CD154 (CD40L) pathway [74].

A study by McAdam et al. implicated murine ICOS in the differentiation of $CD4^+$ T cells [75]. TCR-transgenic T cells differentiated into the Th2 phenotype, expressed significantly more ICOS than cells differentiated towards the Th1 phenotype. Engagement of ICOS is particularly effective in costimulating IL-10 and IL-4 but not IL-2 production. In addition, T cell differentiation, in the presence of an ICOS antagonist, lead to the development of more Th1 than Th2 cells. In support of this, Coyle and colleagues investigated the roles of ICOS and CD28 in the regulation of Th2-mediated mucosal inflammatory responses. They found that both molecules regulate the outcome of the immune response but play separate key roles: CD28 primes T cells whereas ICOS regulates effector responses. The investigators proposed that T cells first become primed by a CD28-B7-dependent mechanism, which lead to upregulation of CXCR5 and subsequent migration to the edge of the B cell rich follicle. ThP (primed) cells then encounter antigen-bearing B cells which present peptides to the antigen-specific ThP cells through B7RP-1. This signal specifically facilitates Th2 cytokine production, thus upregulating CD40 ligand and leading to B cell expansion. In addition, B7RP-1-ICOS interactions would also deliver a signal for the upregulation of CCR3, CCR4 and CCR8. As the ligands for these receptors - eotaxin, MDC and I-309 - are induced during allergic inflammation

from epithelial cells and monocytes and have been implicated in mediating Th2 accumulation in the lungs [76, 77], ICOS-mediated costimulation of effector cells would then provide the signals for these effector cells to migrate from secondary lymph nodes into the airways. However, anti-ICOS or ICOS-Ig administration were given 30 minutes before antigen challenge so the results may be due to antibody competition rather than a downregulation of mucosal inflammation.

However, recent studies are challenging the fact that ICOS-mediated signalling is only associated with Th2 responses. Hancock and colleagues have shown that blockade of ICOS signalling leads to increased heart allograft acceptance and reduced evidence of chronic rejection [78]. Although acute allograft rejection is not Th2-mediated, it is dependent on a Th1-mediated inflammatory response that leads to the destruction of graft tissue. They found that ICOS was induced on host mononuclear cells that were progressively infiltrating the graft. Treatment with anti-ICOS or ICOS-Ig fusion protein suppressed intragraft T cell activation and cytokine expression and prolonged allograft survival in a manner similar to that in $ICOS^{-/-}$ allograft recipients. The combination of anti-ICOS therapy and cyclosporin A led to permanent engraftment.

Another study has implicated ICOS in the immunopathogenesis of EAE [79]. After the induction of experimental allergic encephalomyelitis in SJL mice with proteolipid protein (PLP), brain ICOS mRNA and protein were upregulated on infiltrating $CD3^+$ T cells before disease onset. ICOS blockade during the efferent immune response (9-20 days after immunisation) abrogated disease, but blockade during antigen priming (1-10 days after immunisation) exacerbated disease. It was proposed that deficient IL-13 expression by antigen-specific T cells was the cause of disease exacerbation. ICOS blockade during antigen priming lead to excessive PLP-specific splenocyte proliferation and IFN-γ expression, increased brain IFN-γ mRNA and decreased plasma PLP-specific IgG1.

Thus, it is now clear that manipulation of the ICOS-B&RP-1 pathway has important therapeutic implications beyond the Th2 field.

1.2.4 Antigen Dose and Affinity of Binding to the T Cell Receptor

Early *in vivo* studies showed that both a high and low amount of antigen primed for DTH, whereas a moderate level stimulated antibody production [80]. These results were further supported in BALB/c mice infected with *Leishmania* parasites. Mice infected with very low numbers of the parasites produced a healing DTH response but in mice infected with higher doses, the result was a non-healing antibody response [81]. Fully differentiated Th1 cells are stimulated by moderate but not high doses of antigen, whereas Th2 cells respond over a wider range of concentrations [82]. In the differentiation of naïve $CD4^+$ T cells, Constant et al. demonstrated that low doses of priming antigen favoured development of Th2-like cells, with high doses favouring Th1-like cells [56]. In the same year, Pfeiffer et al. showed that signals delivered via the TCR could influence CD4 T cell differentiation. This was investigated by measuring the strength of binding of the immunodominant peptide of huCol IV to purified MHC class II molecules and by examining the effect of peptide modification on CD4 T cell subset differentiation. Peptide/MHC class II complexes that interacted strongly with the T cell receptor, favoured generation of Th1-like cells, while those that bound weakly favoured priming of Th2-like T cells [47].

Grakoui et al examined the role of the peptide/MHC ligand in CD4+ T cell differentiation into Th1 or Th2 cells using a TCR alphabeta transgenic mouse specific for haemoglobin Hb(54-76)I-Ek. Two altered peptide ligands of Hb(64-76) were found to induce Th2 cytokines at low concentrations and Th1 cytokines at high concentrations. However, the authors argued that the potential to develop into Th1 or Th2 cells was independent of antigen dose. They proposed that the basis for the observed effects on the Th1/Th2 balance shown by the altered peptide ligands and the amount of antigen dose, involved the modification of soluble factors in bulk cultures that were the driving force that polarised the population to either a Th1 or Th2 phenotype [562].

1.2.5 Antigen Structure

Several experiments suggest that certain epitopes can preferentially induce one of the two subsets of Th cells. Liew et al showed that a repetitive peptide of *L. major* selectively activated Th2 cells and enhanced disease progression [83]. In contrast, a recombinant 403 amino acid protein from *L. brasiliensis*, preferentially stimulated human PBMCs to express a Th1-type cytokine profile and to produce IL-12 [84]. The house dust mite allergen Der p 1 can cleave CD23 (the low affinity receptor for IgE) leading to exacerbation of the allergic Th2 response by disrupting the IgE regulatory pathway [85, 86]. Der p 1 can also cleave the α subunit of the human IL-2 receptor (CD25) leading to a disruption in IL-2 production and expansion of IFN-γ-producing Th1 cells, thus biasing Th2 cell induction [87]. The properties of Der p 1 are discussed in greater detail later in this chapter.

1.2.6 Environmental Factors

Environmental factors can take effect before and/or after birth. Early clinical information suggests that Th1 responses are suppressed systemically during pregnancy, and more recent data shows that Th2 cytokines are expressed in the placenta [88]. This may be essential for foetal survival, as inflammatory responses, NK cells and the Th1 cytokines IL-2, IFN-γ and TNF are all harmful for placental maintenance, whereas IL-10 promotes foetal allograft survival. Supporting this, are studies using $CD4^+$ and $CD8^+$ T-cell clones, generated from the decidua of women suffering from unexplained recurrent abortions. These clones showed significantly reduced IL-4 and IL-10 production compared to those generated from the decidua of women with voluntary abortion [89]. In mice, Leishmania major infection is eliminated by a strong Th1 response but persists longer in pregnant mice [90, 91].

Holt et al. suggested that early postnatal exposure to high levels of allergen, exemplified by "birth during the pollen season", maximised the risk for subsequent expression of allergen reactivity to that allergen in adult life [92]. Subsequent studies have shown that initial T cell priming to a variety of allergens can occur prenatally [93, 94] via ingestion or inhalation by the mother [95]. Prescott et al. demonstrated that Th2-skewed responses to common environmental allergens, comprising IL-4, IL-5, IL-6, IL-9 and IL-13 were present in virtually all newborn infants and were

dominated by high level production of IL-10. Szépfalusi and co-workers demonstrated that the transfer of inhalant and nutritive allergens across the human placenta was indeed possible [96]. A second study by the same group showed that foetal T cell priming with inhalant allergens occurs predominantly early in pregnancy, likely before the gestational age of 20 weeks. This conclusion was based on the fact that lymphoproliferative reactivity to inhalant allergens (r*Bet v 1* and r*Phl p 1*) was comparable in cells derived from cord blood samples of term newborns and of unborn (preterm) babies with a gestational age from 20 to 37 weeks.

The decrease of childhood infections such as mycobacterial infection (Th1-orientated) in the developed world as a result of vaccination schedules, is thought to favour Th2 priming, as the cytokines produced during these types of infection are antagonistic to Th2 development [97]. This "hygiene hypothesis" was based on a number of studies *in vitro* and *in vivo*. IL-12 and IFN-γ production by macrophages and NK cells in response to *Mycobacterium tuberculosis,* was able to shift the development of allergen-specific T cells from the Th2/Th0 to the Th1 profile in vitro [98]. These results were substantiated in mice showing that IFN-γ produced during the Th1 response against BCG suppressed the development of local inflammatory Th2 responses in the lung [99]. In Japanese school children, positive tuberculin responses correlated with a lower incidence of asthma, lower serum IgE levels, and cytokine profiles biased towards Th1 responses. Also, expression of atopy in 12 year old children was most prevalent among those who had previously failed to develop Th1-dependent delayed tuberculin hypersensitivity in response to bacillus Calmette-Guérin (BCG) vaccination during infancy [100].

In addition, it has been shown that blockade of endogenous production of IL-12 in naturally resistant murine strains (C3H/HeJ), renders them susceptible to the development of allergen-induced AHR and eosinophilic inflammation [101].

A number of environmental chemicals have been implicated in the prevalence and incidence of allergic diseases during the last few decades [102]. Ozone in particular has been demonstrated as a modulator of the T cell effector response toward the Th2 phenotype [103, 104]. A recent study by Neuhaus-Steinmetz et al, investigated the effect of repeated ozone exposure on the development of immune responses towards ovalbumin. When OVA-sensitised mice were additionally

exposed to ozone, the allergen-specific Th2 response was enhanced in both the IgE-high and IgE-low responder strains [105].

1.2.7 Role of Stat 4 and Stat 6 in Th1/Th2 Development

Differentiation-inducing signals are delivered by the actions of IL-12 and IL-4 on specific receptors expressed by activated T cells, through specific STAT (signal transducers and activators of transcription) factors [106-108]. Cytokine receptors consist of at least two chains that dimerise after binding of the appropriate cytokine. This dimerisation brings together the Janus kinases (JAKs) that are associated in the cytoplasm with the cytokine receptor chains. These JAKs activate each other and phosphorylate the receptor which enables the STATs to bind and in turn become phosphorylated themselves. The phosphorylated STATs dimerise and translocate to the nucleus to initiate gene transcription.

STAT6 is activated by IL-4 and IL-13 to induce Th2 development [109-111]. Studies in STAT6-deficient mice have shown that Th2 cells were unable to develop and production of IgE and IgG1 by B cells was virtually abolished [112, 113]. STAT4 is activated by IL-12 to induce Th1 development [107, 108]. Experiments on Stat 4-deficient T cells have shown that they only produce negligible amounts of IFN-γ [114].

Another transcription factor associated with murine Th2 cells is GATA3 which controls the expression of the IL-5 gene [115]. Conversely, a specific T box transcription factor – T-bet has been found to direct Th1 lineage commitment. T-bet expression was found to correlate with IFN-γ expression in Th1 and NK cells [563].

1.2.8 Markers on Th1 and Th2 Cells

As well as producing different cytokines, polarised human Th1 and Th2 cells preferentially express certain activation markers. For example, CD30 (a member of the TNF receptor family) is mainly expressed in Th2- and Tc2-like cells *in vitro* and *in vivo* [116, 117]; crosslinking of CD30 enhances proliferation of T and B cells. Lymphocyte activation gene 3 (LAG-3), a member of the immunoglobulin superfamily, preferentially associates with Th1-like cells [118]. LAG-3 expression is upregulated by IFN-γ and down regulated by IL-4 [119], in contrast, CD30 is dependent on IL-4 and its expression is therefore prevented in fully differentiated,

IL-4 receptor-lacking Th1 effectors. Other molecules associated with human Th1 cells are CD26 (acts as exopeptidase by cleaving N terminal X-Pro or X-Ala dipeptides from polypeptides), CCR5 and CXCR3. Th1 reactions depend upon IFN-γ-induced CXC chemokines interferon-inducible protein (IP)-10, interferon T cell-alpha chemoattractant (iTAC) and monokine induced by interferon-gamma (MiG), which bind to the chemokine receptor CXCR3. In contrast, CD62L [120], CCR3 (eotaxin and MCP-3 receptor), CCR4 and CCR8 preferentially associate with human Th2 cells during different phases of their differentiation and activation [121]. CD62L binds CD34, GlyCAM-1 and MAdCAM-1 which are expressed on the endothelium. Interaction of CD62L with its ligands mediates specific homing of naïve T cells to lymphoid organs by a rolling mechanism along the endothelium. The c-maf proto-oncogene is a Th2-specific transcription factor that activates the IL-4 promoter in mice [122, 123].

T1/ST2 is constitutively and stably expressed on the surface of murine Th2 cells. This was first shown by Xu et al who used differential display PCR to compare a panel of Th1 and Th2 clones [124]. They also found that an antibody against the extracellular domain of ST2-L lysed Th2 cells in a complement-mediated fashion. A second study by Löhning et al, showed expression of T1/ST2 on Th2 cells at the single cell level and that this expression was independent of IL-4, IL-5 or IL-10 [125]. Mast cells have also been found to express high levels of T1/ST2 [126].

A few studies have suggested that T1/ST2 is more than just a stable Th2 marker in that it is required for Th2 effector responses. Antibodies to ST2-L have been used in a number of disease models. Anti-ST2-L was found to increase resistance to *Leishmania major* infection in BALB/c mice and exacerbated collagen-induced arthritis in DBA/1 mice [124]. Furthermore, administration of either a mAb to T1/ST2 or a T1/ST2 fusion protein attenuated eosinophilic inflammation in the airways in a murine model of Th2-dependent allergic airway inflammation, and suppressed IL-4 and IL-5 production in vivo following adoptive transfer of Th2 cells [125, 127]. However, a study by Hoshino et al disputed this hypothesis. T1/ST2$^{-/-}$ mice were generated and found to exhibit normal Th2 responses to helminth infection and in a mouse model of asthma. In addition, differentiation and function of mast cells was found to be unaffected [128].

1.2.9 Stability of Th1/Th2 cells

In vitro

After one round of stimulation of naive murine TCR-transgenic $CD4^+$ T cells with antigen, APC and IL-12, Th1 cells can be readily converted to Th2 populations by a second round of stimulation with antigen, APC and IL-4 [129, 130]. Analysis of cytokines after one round of stimulation with IL-12 only showed the presence of Th1 cytokines, however, analysis at the single cell level showed the existence of heterogeneous populations, with the majority of cells neither producing IFN-γ nor IL-4 [131]. Thus, this Th1 to Th2 conversion may actually represent either the development of Th2 cells from undifferentiated precursor T cells, or expansion of a small number of contaminating, differentiated Th2 cells. After repeated stimulation under Th1-polarising conditions, this ability to reverse to the Th2 phenotype is lost indicating a stable Th1 phenotype.

In contrast, reversal to a Th1 phenotype after only one round of Th2 stimulation is relatively harder [130]. The Th2 population becomes relatively resistant to phenotype reversal by IL-12 stimulation [129, 132]. However, Th2→Th1 phenotype reversal is not impossible, as shown by Murphy et al [131]; primary populations of Th2 cells gave rise to Th1 cells upon antigenic stimulation in IL-12 but it was noted that numbers of IL-4-producing cells remained the same. They hypothesised that Th1 cells were produced from either uncommitted precursor T cells or the expansion of a rare previously committed Th1 population. This reversibility was only possible after early differentiation to the stable Th2 phenotype; after a number of restimulations in the presence of IL-4, the ability to become Th1-committed was completely lost.

Human populations behave slightly differently. Short-term Th2 cells derived from mitogen-stimulated cord blood cells can still be induced to produce IFN-γ if restimulated in the presence of IL-12 [133]. This suggests that basic differences exist in the differentiation of Th populations or in the regulation of IL-12 receptor expression, in mouse and human culture systems [129, 132, and 134].

In vivo

Th1/Th2 phenotype reversibility *in vivo* is even more resistant to change. For example, in mice infected with *Leishmania major*, the healing Th1 response and non-healing Th2 response are very resistant to reversal even after anti-IFN-γ, anti-IL-4 and IL-12 treatment. Reversal (Th2→Th1) is only possible within the first week of infection or after treatment with a combination of IL-12 and a parasiticidal drug [135]. Recently, Janssen et al. showed that an established *in vivo* Th2 response could be modulated by treatment with a Th1-skewing peptide, which resulted in decreased airway eosinophilia and antigen-specific IL-4 and IL-5 production [136].

1.2.10 Adoptive Transfer

Reversibility of Th1/Th2 populations after adoptive transfer is possible in the recipient, even after reversibility in the host is no longer possible. Transfer of LN cells from a non-healing Th2 BALB/c mouse into T and B cell-deficient C.B-17 scid mice along with anti-IL-4, resulted in a stable dominant protective Th1 response [137]. This can probably be attributed to the existence of different microenvironments between the host and recipient. Thus, adoptive transfer experiments have the potential for reversing Th1/Th2 responses that are difficult to reverse *in situ*.

1.3 $CD8^+$ T Cells

$CD8^+$ T cells have been classically associated with cytolytic function. All viruses and some bacteria multiply in the cytoplasm of infected cells and thus, are not accessible to antibodies. A mechanism has therefore evolved in which infected cells are programmed to undergo apoptosis by cytotoxic T cells. The cytotoxic T cell recognises foreign peptides bound to MHC class I molecules that are transported to the surface of an infected cell. Cytotoxic T cells release a number of effector molecules to initiate apoptosis of the target cell. The calcium-dependent release of specialised lytic granules upon antigen recognition is the initial step. These granules are modified lysosomes that contain a number of proteins, that function once released. Perforin polymerises to form transmembrane pores in the target cell

membranes, which allows water and salts to pass rapidly into the cell. Once the integrity of the cell membrane is destroyed, the cell dies rapidly. Granzymes, which comprise of serine proteases, activate apoptosis once in the cytoplasm of the target cell. The granzymes are unable to defragment the DNA directly but act by cleaving the ubiquitous cellular enzyme CPP-22. This is believed to play an essential role in the apoptosis of all cells. As well as the destruction of cellular DNA, viral DNA is also degraded which prevents the assembly of virions and thus spread of the virus to neighbouring cells. The apoptotic cells are then phagocytosed by other cells in the vicinity [138, 139].

Cytotoxic T cells are very discriminate in their killing so 'innocent' bystander cells are spared. By orientating their Golgi apparatus and microtubule-organising centre, the secretory apparatus is focused at the point of contact with the target cell. Thus, the cytotoxic T cells kill target cells one by one in this manner. Targets are killed rapidly because cytotoxic proteins are stored in inactive forms in the lytic granules. Cytotoxins are synthesised and located in the lytic granules on the first encounter of a naïve cytotoxic precursor T cell with specific antigen. These stocks are constantly replenished by ligation of the T-cell receptor which induces *de novo* synthesis of the proteins, enabling the CD8 T cell to kill many targets in succession.

A calcium-independent mechanism of killing also exists for $CD8^+$ and some $CD4^+$ T cells. This involves the binding of Fas in the target cell membrane by Fas ligand, which is expressed in the membranes of activated cytotoxic T cells and Th1 cells. Activation of Fas also leads to apoptosis in the target cell [140].

Cytotoxic T cells also contribute to host defence by releasing IFN-γ, TNF-α and TNF-β [141]. IFN-γ directly inhibits viral replication, induces the upregulation of more MHC class I expression on APCs and leads to macrophage activation, recruiting them to sites of infection as effector cells and APCs. TNF-α and TNF-β are also involved in macrophage activation and can kill some target cells through their interaction with TNFR1.

Studies have demonstrated that $CD8^+$ T cells can be sub-divided into Tc1 and Tc2 subsets in humans and mice [551, 552]. These $CD8^+$ T cell subsets can be derived under similar conditions as those needed to generate Th1 and Th2 subsets: IL-12 and IFN-γ promote Th1 and Tc1 induction whilst IL-4 induces Th2 and Tc2

generation. Evidence suggests that Tc1 and Tc2 cells are stable and retain their cytokine profile [142].

CD8 T cells have been identified *in vivo* in a number of infectious diseases. Tc2-like cells have been isolated from patients with lepromatous leprosy and from HIV-infected individuals with Job's-like syndrome (high IgE levels) [143], whereas Tc1-like cells have been isolated from patients with tuberculoid leprosy. Both Tc1 and Tc2 subsets have cytotoxic activity but this differs depending on the disease model [144] and may depend on cytokine exposure prior to the cytotoxicity assay [145].

1.3.1 $CD8^+$ T Cells in Allergic Inflammation

Earlier in this chapter, the effect of viral and bacterial infections on the induction of a Th1 response was discussed. However, viral/microbial infections and/or their products may have bidirectional effects on the development of allergy and asthma.

McMenamin et al. demonstrated in aerosol-exposed rats during the early sensitisation phase, that $CD8^+$ IFN-γ-producing cells may provide the initial source of T cell-derived IFN-γ and thus, play a critical role in preventing the induction of allergic responses [146]. Early studies with $CD8^+$ T cells have implicated them in the suppression of IgE production. Renz et al showed that allergen-reactive CD8 T cells from sensitised mice when adoptively transferred to pre-sensitised recipient mice, caused a significant reduction specific serum IgE [147]. Furthermore, measurement of *in vitro* Ig production indicated that mononuclear cells from recipients of CD8 cells ($CD8^{OVA} > CD8^{PBS}$) produced less IgE and IgG1 antibodies, whereas IgG2a production was enhanced. Airway hyperresponsiveness and immediate-type skin reactions were also prevented. This suggested that $CD8^+$ T cells play a role in the negative regulation of IgE production and AHR. However, other studies have demonstrated that $CD8^+$ T cells have the potential to stimulate IgE-class switching and promote allergic inflammation. Rats depleted of $CD8^+$ T cells or nude rats reconstituted with $CD4^+$ but not $CD8^+$ T cells prior to immunisation, failed to mount an IgE response [148]. *In vitro* studies analysing T cell subsets in allergic patients revealed that twice as many $CD4^+$ T cells and six times as many $CD8^+$ T cells

produced IL-4 compared with non-allergic controls. Other studies have demonstrated that IL-13 was the major IgE-synthesis-inducing cytokine from Th1 and $CD8^+$ T cells [149]. Furthermore, $CD8^+$ T cells have been observed at sites of allergic inflammation [150], although they were not the major subset present.

Hamelmann et al. studied the role of $CD8^+$ T cells in allergic airway sensitisation. The depletion of $CD8^+$ T cells before aerosolised OVA sensitisation in BALB/c mice, prevented the development of airway hyperresponsiveness despite the production of allergen-specific IgE. The requirement for the induction of AHR was confirmed in cell transfer experiments; transfer of nonprimed $CD8^+$ T cells restored the ability to develop AHR in CD8 T cell-depleted mice [151]. However, a later study by Haczku using Brown-Norway rats found that purified $CD4^+$ T cells from sensitised donors induced AHR in naïve recipients, while sensitised $CD8^+$ and naïve $CD4^+$ cells failed to do so [152].

Virus-specific $CD8^+$ T cells have also been reported to induce a switch to IL-5 production and induce eosinophilia [153]. Transgenic mice expressing an H-$2D^b$-restricted TCR specific for the epitope 31-44 of the lymphocytic choriomeningitis virus (LCMV), were immunised with OVA to produce a Th2 response and then challenged intranasally with LCMV glycoprotein 33-41 peptide. The bystander $CD4^+$ response to OVA was able to switch the virus peptide-specific $CD8^+$ T cells to IL-5 production and furthermore, when those IL-5-producing $CD8^+$ cells were challenged via the airways with virus peptide, significant eosinophilia was induced. These results provided a possible link between virus infection and exacerbation of asthma. Production of IL-5 by virus-reactive $CD8^+$ T cells is dependent on $CD4^+$ bystander T helper activity. Virus peptide-reactive $CD8^+$ T cells were found to secrete IL-5, leading to eosinophilia when mice were challenged via the airways.

A number of respiratory viruses can exacerbate asthma in humans, the most common being rhinovirus. This effect has now been demonstrated in animal models, with Suzuki et al reporting that an infection with influenza A virus lead to increased airway responsiveness and allergen-specific IgE production in aerosolised, antigen-exposed mice [154]. Other studies reported an increase in OVA-specific IgG1 antibodies leading to anaphylactic shock after OVA-induced sensitisation was combined with influenza A virus or RSV infection in mice [155]. Infections with

RSV were also found to increase allergen-induced airway hyper-reactivity in mice [156].

Perhaps it is not surprising that considering the existence of different Tc subsets, $CD8^+$ T cells have been implicated in both the moderation and exacerbation of allergic inflammation.

1.4 Allergy and Asthma

When an adaptive immune response occurs in an exaggerated or inappropriate form, the term hypersensitivity is applied. Hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately, and sometimes cause inflammatory reactions and tissue damage. Hypersensitivity is not manifested on first contact with the antigen, but usually appears on subsequent contact. Type I hypersensitivity is characterised by an allergic reaction that occurs immediately following contact with the antigen, referred to as the allergen. The term 'allergy' means 'changed reactivity' of the host when meeting an 'agent' on a second or subsequent occasion. In recent years 'allergy' has become synonymous with type I hypersensitivity.

For an allergic response to occur, a naïve individual first becomes sensitised by mounting an antibody or T cell response to the allergen. This adaptive response takes time and does not usually cause any symptoms. Allergic responses are characterised by the enhanced ability of B cells to produce IgE antibodies, driven by the production of IL-4 and IL-13 by Th2 cells. When a sensitised individual is challenged with antigen, a bimodal response ensues consisting of a classical Type I hypersensitivity reaction followed by a type IV reaction with Th2 cytokine characteristics. The early phase response peaks at 10-15 minutes and resolves after approximately 1 hour. It is mediated by the cross-linking of allergen specific IgE on the surface of mast cells.

The late phase response begins at about 3 hours, peaks at 6-9 hours and resolves by 24 hours. Once IgE has bound to FcεRI on mast cells and basophils, degranulation can be triggered by IgE crosslinking. This relies upon a degree of conformational integrity within the allergen molecule (or fragment) and the presence

of at least two contiguous epitopes in order to bridge adjacent IgE molecules. This causes an influx of calcium ions into the mast cells and basophils resulting in their degranulation. Degranulation results in the release of preformed mediators, the major one in man being histamine. There is also an induction of synthesis of newly formed mediators from arachidonic acid, leading to the production of prostaglandins and leukotrienes, which have a direct effect on the local tissues. In the lung they cause immediate bronchoconstriction (which is mediated by eosinophil infiltration to the site of allergen challenge), mucosal oedema and hypersecretion leading to asthma [157, 158].

Most allergens are relatively small, highly soluble protein molecules that are inhaled in desiccated particles such as pollen grains or mite faeces. The allergen elutes from the particle because it is readily soluble and diffuses into the mucosa. Typical symptoms of an allergic reaction include rhinitis (runny nose and sneezing), asthma and in severe cases, systemic anaphylaxis in response to an insect bite or sting, or even a food product in highly sensitised individuals. Once an individual is sensitised, each subsequent exposure to the allergen often leads to worsening of symptoms because antibody levels and effector T cell responses increase.

Other common hypersensitivity reactions are termed type IV or delayed hypersensitivity because they take time to manifest. Examples of these reactions are contact dermatitis, graft rejection, rheumatoid arthritis and diabetes mellitus. The reactions are T cell mediated either by inflammatory (Th1) cells that are mainly induced by macrophages, or cytotoxic $CD8^+$ T cells.

Over the years, a number of studies have supported the hypothesis that allergen-specific Th2 cells have a pathogenic role in allergic disorders:

- Allergens preferentially expand Th cells showing a Th2-like profile [159, 160]
- Th2-like cells accumulate in the target organs of allergic patients [161, 162]
- Allergen challenge results in local activation and recruitment of allergen-specific Th2-like cells [163, 164]
- Atopic asthma is associated with activation in the bronchi of the IL-3, 4 and 5 and GM-CSF gene cluster, a pattern compatible with predominant activation of the Th2 like T cell population [564]

development of allergen-induced allergic airway responses. Furthermore, the adoptive transfer of Th2 clones into the lungs of naïve mice induced AHR [176].

The excessive production of airway mucus glycoproteins is a consistent finding in the lungs of asthmatic subjects, especially in those that have died as a result of asthma [177]. IL-4 has been directly implicated in mucus production as shown in IL-4 transgenic mice, which exhibit profound increases in mucus-containing cells in the airway epithelium [178]. In contrast, blockade of the IL-4 receptor results in ablation of mucus-containing cells [179].

Goblet cell hyperplasia is thought to be mediated by mast cell and eosinophil recruitment since these effector cells release the potent mucus secretogues LT, hydroxyeicosatetraenoic acid (15-HETES) and platelet-activating factor (PAF).

1.4.1 IL-4

IL-4 is a key cytokine in the development of allergic inflammation. It is associated with induction of the ε isotype switch and secretion of IgE by B lymphocytes [565]. IL-4 further enhances IgE-mediated immune responses by upregulating IgE receptors on the cell surface: the low affinity receptor (FCεRII; CD23) on B lymphocytes and mononuclear phagocytic cells and the high affinity IgE receptor FCεRI on mast cells and basophils [566]. The role of mast cells and basophils in the allergic response is discussed on page 45.

An additional mechanism by which IL-4 contributes to airway obstruction in asthma is through the induction of mucin gene expression and the hypersecretion of mucus [567]. IL-4 increases the expression of eotaxin and other inflammatory cytokines from fibroblasts that might contribute to inflammation and lung remodelling in chronic asthma [568]. IL-4 induces the expression of VCAM-1 in the asthmatic lung thus promoting cellular inflammation [569]. Through the interaction of VCAM-1, IL-4 is able to direct the migration of T lymphocytes, monocytes, basophils and eosinophils to inflammatory loci. In addition, IL-4 inhibits eosinophil apoptosis and promotes eosinophilic inflammation by inducing eosinophil chemotaxis and activation through increased expression of eotaxin (discussed in more detail on page 52) [570].

- Successful specific immunotherapy is associated with the down-regulation of allergen-reactive Th2 cells and/or up-regulation of allergen-reactive Th1 cells [165, 166] [167]

Asthma is a chronic inflammatory disease of the lung that has increased in prevalence in recent years. It is characterised by the continued presence of increased numbers of Th2 lymphocytes, eosinophils, neutrophils and mast cells, along with epithelial desquamation, goblet cell hyperplasia and thickening of the submucosa [169]. This in turn, leads to variable airflow obstruction and AHR (airway hyperresponsiveness) [170].

The allergic asthma reaction can be divided into an early (within minutes) bronchospastic response followed by a late (hours) inflammatory response. The immediate reaction can be characterised by rapid onset of mucosal oedema, increases in airway smooth muscle tone and airway narrowing due to the release of vasoactive mediators by mast cells. The late phase response is associated with the migration of neutrophils, eosinophils and lymphocytes from the blood into the lung parenchyma and airway epithelium. Mast cells have also been implicated in the late phase response. Support for this has come from a study in which the administration of sodium nedocromil, a mast cell stabiliser, was found to inhibit both the early and late phase responses [172]. They may also act indirectly by the release of cytokines such as IL-4 which leads to an increase in Th2 lymphocytes and eosinophil infiltration [173].

Studies in asthmatics have shown an increase in T cell activation, with increased expression of the IL-2 receptor, MHC class II antigens and very late activation antigen-1 (VLA-1) in blood, BAL and bronchial biopsies [174]. As mentioned earlier, Th2 cells produce IL-4, IL-13, IL-5, IL-9, IL-6 and IL-10 which are important in the stimulation of IgE production, mucosal mastocytosis and eosinophilia.

A link between $CD4^+$ T cells in the development of AHR was first demonstrated in 1994 by Gavett and colleagues [175]. In a murine model of ovalbumin sensitisation, depletion of $CD4^+$ T cells in sensitised mice by specific monoclonal antibodies, prior to local lung antigen challenge, prevented the

The ability of IL-4 to drive differentiation of naïve Th0 lymphocytes into Th2 lymphocytes has already been discussed. IL-4 is also able to prevent apoptosis of T lymphocytes. This inhibition of apoptosis of is thought to be mediated by the ability of IL-4 to maintain levels of the survival-promoting protein Bcl-2 in T cells [571]. It was found that the co-culture of T cells with IL-4 downregulates Fas expression on the cell surface thus preventing apoptosis through the interaction of Fas (CD95) and Fas ligand.

1.4.2 IL-5

IL-5 is essential for the maturation of eosinophils in the bone marrow and their release into the blood [545]. In humans, IL-5 acts only on eosinophils and basophils, in which it causes maturation, growth, activation and survival [572,573]. This specificity occurs because only those cells possess the IL-5 receptor. The role of IL-5 in eosinophilia is discussed in more detail on page 51.

1.4.3 IL-9

IL-9 is a T cell derived cytokine with pleiotropic effects on various cell types [180]. Preferential production of IL-9 by Th2 cells has [181, 182] been implicated in the *in vitro* stimulation of activated T cells [183], enhancement of immunoglobulin production in B cells [184, 185] and the proliferation and differentiation of mast cells [186]. In addition, IL-9 has been shown to effect IgE-mediated responses by up-regulating the α chain of the high affinity IgE receptor [187].

IL-9 has been proposed as a candidate gene for asthma. The gene for IL-9 is located within human chromosome 5q31-33 which is associated with asthma and bronchial hyperresponsiveness [188, 189]. Studies with inbred strains of mice demonstrated that bronchial hyperresponsiveness is genetically linked to chromosome 13, which shows homology with human chromosome 5q31-33 [190]. The hyporesponsive B6 strain of mice express undetectable levels of IL-9 protein in the steady state but the hyperresponsive D2 strain of mice have significant IL-9 protein levels in the lung. Thus, there appears to be a tight genotype-phenotype correlation. In naïve B6 mice, intratracheal instillation of recombinant murine IL-9 induced eosinophilia with an elevation in total serum IgE [191]. Furthermore,

transgenic mice over-expressing IL-9, showed enhanced eosinophil infiltration, elevated total serum IgE, mast cell hyperplasia and airway hyperresponsiveness [192, 193]. In humans, the *in vivo* expression of IL-9 was found to be significantly up-regulated in bronchial biopsy specimens obtained from subjects with asthma, compared with those from controls and from subjects with non-atopic lung disease [194].

IL-9 can stimulate mucin transcription and the production of chemokines by respiratory epithelial cells. The expression of IL-9 in transgenic mice resulted in the upregulation of eotaxin and MCP-5, which have chemotactic activity on eosinophils [195]. Also, Levitt et al have shown an induction of IL-5Rα by IL-9 in mice suggesting that IL-9 has a direct effect in eosinophilia [191]. Mucus overproduction is often observed in airway inflammation and is the leading cause of airway obstruction in asthma [196]. Longphre *et al* blocked the ligand-receptor interactions of IL-5, IL-13 and IL-9 in dog airway fluid and found that only IL-9 stimulated mucin synthesis [197].

1.4.4 IL-13

IL-13 consists of 132 amino acids and has a molecular mass of 12 kDa. The gene for human IL-13 is located on chromosome 5q 31 in the same region as the genes encoding IL-3, IL-4, IL-5, IL-9 and GM-CSF [198]. IL-13 shows 25% homology with IL-4 and shares some structural characteristics and functional properties with the cytokine. Both the IL-4R and IL-13R share the IL-4Rα chain and promote STAT 6 activation.

IL-13 production was found to be independent of IL-4 as observed in IL-4 deficient mice, after challenge with *Onchocerca volvulus* antigen [199]. IL-13 in contrast to IL-4 does not support the proliferation of activated human and mouse T cells and failed to induce the differentiation of naïve $CD4^+$ cord blood T cells towards a Th2 phenotype [133].

The expression of CD23, CD71 (on activated leukocytes), CD72 (on B cells), sIgM and class II MHC antigens can all be upregulated by IL-13. In addition, IL-13 has growth-promoting and anti-apoptotic effects on normal B cells activated by anti-

IgM or anti-CD40 mAbs. The production of IgM, IgG and IgA can also be enhanced by IL-13.

The physiological effects of IL-13 are contradictory. On one hand, IL-13 has been shown to induce IgE synthesis independently of IL-4, [200] and unlike IL-4, is produced by naïve $CD45RA^+$ T cells, implying that it is important for the initiation of IgE production. The role of IL-13 in allergic asthma has also been examined [201, 574]. Mice were immunised intraperitoneally with ovalbumin and subsequently challenged intratracheally with the protein in order to induce an allergic phenotype. IL-13 production was blocked by the administration of a soluble IL-13α2-IgGFc fusion protein, which specifically bound and neutralised IL-13, subsequently causing a complete reversal of allergen-induced AHR. As well as allergic responses, parasitic worm infections are also characterised by the induction of a Th2 response. Originally, IL-4 was thought to be the major cytokine responsible for worm clearance but more recent studies using *Nippostrogylus brasiliensis* (a gastrointestinal nematode) infected $IL4^{-/-}$ mice, have demonstrated that these animals were able to expel the parasite [202], suggesting that another T cell mediator is required for efficient worm clearance. A study using $IL\text{-}13^{-/-}$ mice, showed that these animals were unable to clear the *N. brasiliensis* infection efficiently, even though a strong Th2 response was observed [203]. Furthermore, treatment of the infected $IL\text{-}13^{-/-}$ mice with exogenous IL-13, resulted in a decreased worm burden. $CD8^+$ T cells have been shown to produce IL-13 [149], which supports the view that these cells are responsible for exacerbation of asthma following viral infection [153]. Furthermore, IL-13 can induce VCAM-1 expression on endothelial cells, which results in the adhesion and subsequent extravasation of T cells, monocytes and eosinophils to sites of allergic inflammation [204].

IL-13 is also capable of antagonising a Th1 response. IL-13 has the capacity to downregulate the production of RANTES *in vitro* by airway-derived smooth muscle and endothelial cells [205]. *In vivo*, IL-13 inhibits LPS-induced lethal shock, collagen-induced arthritis, EAE and diseases in which TNF-α produced by macrophages plays a major role [206, 207].

1.4.5 Antigen Presentation in the Airways

Dendritic cells are bone marrow-derived professional APCs and are probably the most important antigen presenting cell in the airways. The DCs form an extensive network below the basement membrane of the airway epithelium that ensures accessibility to inhaled antigens [208, 209]. The density of DCs is highest in the upper airways with a decline in number further down the airways [210], which suggests that these APCs may play a sentinel function in detecting the presence of foreign antigens [211]. Dendritic cells are stellate with many fine dendrites. This shape combined with excellent motility are the characteristics that make DCs excellent at capturing antigens and selecting antigen-specific T cells. DCs display large amounts of MHC-peptide complexes on their surface which are typically 10-100 times higher on DCs than on other APCs like B cells and monocytes. Chemokine receptors expressed by immature DCs, promote their migration to inflamed tissues, where antigens are captured and maturation is induced. Maturing DCs upregulate CCR7 which drives their migration to the T-cell areas of the draining lymph nodes where antigen is presented to naïve T cells [212]. Lambrecht et al, reported a requirement for DCs in the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitised mice [213].

DC numbers in the airways have been shown to be elevated in a number of asthmatics compared to normal controls [214] and also, the proportion of DCs expressing the alpha subunit of the FcεR1 is significantly increased in asthmatics [215]. Steroid treatment dramatically inhibits the recruitment of DCs into the respiratory tract epithelium during acute inflammation [216].

Another cell type implicated in airway inflammation is the alveolar macrophage (AM) [217, 218]. Alveolar macrophages from asthmatic patients express an enhanced capacity to present antigen to autologous T-lymphocytes [219], than those from normal subjects, and also express elevated levels of ICAM-1 and LFA-1 [220]. Stimulated AMs from asthmatics produce more GM-CSF, TNF-α, IL-8 and leukotrienes than AMs from nonasthmatics [221, 222], and it has been shown that AMs influence the production of IL-5 by $CD4^+$ T cells [223, 224]. However, resident pulmonary alveolar macrophages (PAM) have been shown to play an important role

in the maintenance of immunological homeostasis in the lung via downmodulation of local T cell responses in the steady state [225].

1.4.6 Eosinophils

Eosinophils are present in relatively low numbers in healthy individuals, but their numbers increase dramatically in certain diseases, particularly parasite infections and allergic conditions [226]. The recruitment of eosinophils into the lung followed by their release of chemotactic factors results in chronic thickening and inflammation of the airways. This recruitment of eosinophils to sites of allergic inflammation is a complex process and is mediated by a number of cytokines and chemokines. The induction of airway eosinophilia is also dependent on CD80 (B7-1) costimulation [64].

IL-5 is probably the most important cytokine to be implicated in eosinophil development, migration and function [227-229]. Experiments with IL-5 deficient mice have established that blood and airway eosinophilia is dependent on IL-5 [230]. IL-5 specifically facilitates the terminal differentiation and proliferation of eosinophils [227], as well as the survival, viability, and antibody-dependent cytotoxicity of mature eosinophils [228]. In addition, IL-5 can induce migration of mouse and human eosinophils [229].

Activation of the endothelium is an important step in the inflammatory process. The endothelium can be activated by a variety of proinflammatory mediators such as IL-1β [231, 232] and TNF-α [233] to induce the expression of ICAM-1. VCAM-1 is induced primarily by IL-4. The initial attachment of eosinophils is thought to be mediated through E-selectin and VCAM-1/VLA-4 interaction [234, 235]; and the upregulation of adhesion molecules such as ICAM-1 and VCAM-1 enhances the attachment of eosinophils and thus exacerbates airway inflammation. The rolling of eosinophils on the endothelium is mediated primarily by P-selectin. After cellular activation by exposure to chemoattractants such as platelet-activating factor [236] or eotaxin, [237-239] eosinophils adhere firmly to the endothelium through adhesion molecules of the integrin family. These include the CD18 family (β_2 integrins) and very-late-antigen-4 (VLA-4) molecules (β_1 integrins). The β_2 integrins interact with intercellular adhesion molecule 1 (ICAM-1)

on endothelial cells, whereas the β_1 integrins interact with vascular-cell adhesion molecule 1 (VCAM-1). The subsequent transendothelial migration process relies heavily upon ICAM-1/CD11/18 [240]. Upregulation of CD11b and shedding of L-selectin during transendothelial migration was also observed [232].

IL-3 and GM-CSF are also implicated in the production and effector function of eosinophils [241, 242]. IL-3 and GM-CSF stimulate bone marrow progenitors *in vitro* to form colonies of cells that include eosinophils. They also promote inflammatory changes in eosinophils characterised by prolonged viability, enhanced functional properties and hypodensity. Hypodense eosinophils have been found in the peripheral blood of patients with chronic helminthic infections, bronchial asthma and hypereosinophilic syndrome. Upon activation, the eosinophil releases preformed toxic cationic proteins such as major basic protein, eosinophil cationic protein [243] and eosinophil peroxidase [244]. In addition, the eosinophil generates oxidative products, hyperhalous acids, lipid mediators (platelet activating factor and leukotriene C_4) and cytokines (IL-3, IL-5 and GM-CSF) that all contribute to the eosinophil's proinflammatory functions. The production of PAF and leukotriene C_4 are dependent on expression of phospolipase A_2 by eosinophils [245]. These are characteristics of eosinophils that make them excellent candidates for defending the host against invading pathogens but contribute enormously to the symptoms of chronic inflammatory diseases such as asthma.

Eosinophil movement within inflamed tissues is promoted by IL-5 in conjunction with the chemokines – RANTES, MIP-1α (macrophage inflammatory protein) [246], MCP-3 (monocyte chemoattractant protein) [247], lipid mediators such as PAF (platelet-activating factor) and leukotriene B_4 [236, 248, 249]], and eotaxin.

Eotaxin was first identified in 1994 by Jose *et al* when eosinophil chemoattractant activity was purified from the BAL fluid of sensitised guinea pigs after challenge with ovalbumin [239]. Microsequencing revealed a novel 73 amino acid C-C chemokine that exhibited 53% homology with human MCP-1, 44% with guinea pig MCP-1, 31% with MIP-1α, and 26% with human RANTES. Human eotaxin was cloned shortly after [250, 251] and more recently, another C-C

chemokine was cloned which showed chemotactic activity for eosinophils and basophils. This chemokine was termed eotaxin-2 and only exhibited 39% homology with eotaxin-1 and was much less potent [252, 253].

Eotaxin is constitutively expressed in a number of tissues and may regulate the basal tissue homing of eosinophils. On exposure of a tissue in a sensitised animal to allergen, there is an upregulation of eotaxin mRNA and an early appearance of protein. However, eotaxin will be unable to induce eosinophil recruitment to any extent if circulating cell numbers are low. Collins *et al* proposed that the secretion of lL-5 acts in an autocrine fashion in that it (IL-5) circulates in the blood and stimulates the release of a rapidly mobilizable pool of bone marrow eosinophils [237]. The synergism of IL-5 and eotaxin will then induce a rapid recruitment of eosinophils from the bone marrow into the affected tissue [254]. The specificity and potency of eotaxin for eosinophils are the result of its high affinity interaction with the β-chemokine receptor, CCR3, which is highly expressed in human and mouse eosinophils [255, 256].

Eosinophilic infiltration of tissues is usually followed by elimination of these cells by apoptosis. Inhibition of apoptosis of eosinophils by certain cytokines (IL-5, GM-CSF) increases the survival of eosinophils and thus, exacerbates eosinophilia. Recent work has suggested that Bcl-x_L (an anti-apoptotic protein) may be involved in the anti-apoptotic pathway mediated by cytokines in eosinophils [257].

1.4.7 IgE

The presence of IgE was first identified in 1966 by Ishizaka et al. [258]. IgE is a 188 kDa immunoglobulin with a very short half-life in serum of two days [259]. In normal, non-atopic individuals, IgE is present at a concentration of 5 $x10^{-5}$ mg/ml in serum, but in allergic subjects or those with a parasitic infection, the levels are significantly elevated.

IgE, like IgM, lacks a hinge region but each isotype has an extra heavy chain domain and also show a wide distribution of N-linked carbohydrate groups [260]. IgE can bind to two classes of receptor – the high affinity (FcεR1) and low affinity receptor (FcεRII, CD23). CD23 is found on activated B cells, but several cell types including, T cells, eosinophils, follicular dendritic cells, neutrophils and

macrophages, also express CD23 [261]. As mentioned before, $CD4^+$ T cells in atopic individuals secrete high levels of IL-4 which promotes IgE synthesis by B cells. The synthesis of IgE is thought to be controlled by a feedback inhibition mechanism that is mediated by IgE-containing complexes binding to CD23 on B cells, which prevents further IgE synthesis [262]. In CD23 knockout mice, the feedback inhibition is absent and thus, IgE levels are increased in response to T cell-dependent antigens [263]. CD23 cleavage from the cell surface is thought to involve proteolysis but the mechanism has not been fully elucidated [264]. Its cleavage results in the formation of soluble trimeric CD23 (sCD23) molecules that retain their ability to bind IgE and thus exacerbate allergic symptoms. Monomeric forms of CD23 can be inhibitory.

As well as binding to specific IgE receptors, IgE can bind to other structures, e.g. FcγReceptors [265] and Mac-2 [553]. Receptor-bound IgE has an extended half-life.

1.4.8 Mechanism of Class Switching

IgE production by B cells is preceded by an isotype class switch from IgM to IgE. Studies *in vitro*, have demonstrated that stimulation of B cells with anti-IgM antibodies or LPS in the presence of IL-4 is sufficient to induce the class switch to IgE [266]. The two signals required for the switch to IgE production are delivered by T cells to B cells through a complex series of interactions. Synthesis of IgA and IgM antibodies by naïve B cells on initial exposure to antigens, is followed by an expansion of memory B cells recognising antigen fragments. On subsequent exposure to the same antigen, the IgM on the B cell surface binds the antigen and the antigen-receptor complex is internalised. Endosomal processing leads to antigenic peptide fragments associated with MHC class II molecules being presented to T cells. Subsequent recognition by the TCR complex leads to two crucial events:

1) secretion of IL-4 and IL-13 and
2) expression of the CD40 ligand.

Signals transmitted following binding of IL-4 to its receptor on the B cell induces transcription of the Cε heavy chain gene locus [267]. CD40L on the T cell, engages CD40, a 50 kDa integral membrane glycoprotein member of the TNF receptor

superfamily, that is constitutively expressed on B cells, macrophages, dendritic cells and endothelial cells [268]. This oligomerisation of CD40 triggers deletional switch recombination to IgE. Expression of B7 (CD80) on B cells is increased following CD40 activation and this serves as a co-receptor for CD28 on T cells, leading to optimal T cell activation and secretion of cytokines [269].

A number of studies have revealed the importance of CD40-CD40L interactions in isotype switching. It was shown that monoclonal antibodies directed against CD40, eliminated the need for T cells in IL-4-driven isotype switching to IgE ***in vitro*** [270]. Patients that had X-linked hyper-IgM syndrome (X-HIM) were deficient in CD40L and were found to have defects in isotype switching and thus lacked serum IgG, IgA and IgE [271, 272]. Similarly, mice with a targeted disruption of the CD40L gene [273], lacked serum IgE and failed to undergo isotype switching *in vivo* and *in vitro* following immunisation with T cell-dependent antigens but were capable of mounting IgM and IgG responses to T cell-independent antigens.

High levels of antigen-specific IgE is obviously detrimental in allergic disease but in parasitic infections, the presence of IgE is very beneficial. Mast cells have been implicated in the clearance of parasites. This has been demonstrated in w/w^v mutant mice, which have a profound mast-cell deficiency caused by a maturation of the gene c-kit [274]. These mutant mice had impaired clearance of the intestinal nematodes *Trichinella spiralis* and *Strongyloides* species. This clearance was impaired to an even greater extent in IL-3-deficient w/w^v mice because they failed to produce basophils as well as lacking functional mast cells.

1.4.9 Neurotrophins

Recently, neurotrophins have been implicated as mediators of inflammation and hyperresponsiveness. Neurotrophin receptors are expressed on nerve cells as well as on immune cells, including monocytes, mast cells, B cells and T cells. Nerve growth factor (NGF) has several immunological functions such as induction of mast cell degranulation, induction of cytokine synthesis, and regulation of antibody production [275]. Additionally, NGF is a chemoattractant and activation factor for eosinophils [276]. In one study, high levels of NGF that correlated with high serum IgE levels and amounts of eosinophilic cationic protein, were found in patients with

severe allergic bronchial asthma. Furthermore, NGF serum levels correlated with total IgE antibody titres [277]. A more recent study found a significant increase in the neurotrophins NGF, BDNF (brain-derived neurotrophic factor) and neurotrophin-3 in the BAL fluid of human volunteers 18 hours after segmental allergen provocation [278].

1.4.10 Genetic Background

The possibility that atopic subjects have a genetic dysregulation at the level of Th cell-derived IL-4 is supported by several observations. First, $CD4^+$ T cell clones from atopic individuals produce noticeable amounts of IL-4 and IL-5 in response to bacterial antigens, such as PPD and streptokinase, that usually evoke responses with a Th1-like cytokine profile in non-atopic individuals [279]. Second, atopic donors have a higher frequency of IL-4 producing T cells than normal subjects [280]. Also, T cell clones generated from neonatal cord blood lymphocytes from new-borns with atopic parents showed higher levels of IL-4 than those born with non-atopic parents [281].

Since 1989 when Cookson and coworkers first reported that they had identified a locus containing an atopy gene on chromosome 11q [282], there have been important advances in incriminating specific loci in the human genome that harbour genes that contribute to asthma and allergy [283 for review].

Atopy, (ie. the predisposition of some individuals to produce IgE after contact with low levels of innocuous allergens), has been linked to the FcεRIβ gene found on chromosome 11 (the 11q13 region) [282]. Mutations in these genes could lead to increased signal transduction after allergen binds to IgE, and consequently to increased secretion of IL-4. One such mutation (Leu-181) was associated with atopy [284] and asthma [285] in some populations but not in others.

On chromosome 5q there are many candidate genes for asthma and allergy such as IL-4 and β_2-andrenergic receptor genes. A specific polymorphism in the IL-4 gene (IL-4 C-589T) has been identified in a region of the gene that binds transcription factors and influences gene expression [286]. This polymorphism has been shown to correlate with high serum IgE levels and enhanced IL-4 gene

expression. A number of variants in the β_2-adrenergic receptor gene have been identified that alter receptor function. The most prevalent of these are the Arg-16 → Gly and the Gln-27 → Glu polymorphisms, which influence the downregulation of the receptor in response to the agonist. The Gly-16/Gln-27 haplotype was associated with bronchial hyperresponsiveness (BHR) and asthma severity [287]. The Gln-27 allele was associated with high total serum IgE [288].

Chromosome 5q also contains genes for IL-5, IL-9, IL-13, GM-CSF [289, 290] and CD14 [291]. CD14 is found on the surface of monocytes and macrophages and is also present in a soluble form (sCD14). CD14 acts as the high-affinity receptor for bacterial LPS (lipopolysaccharide, also known as endotoxin) and is a key molecule in the initiation of the nonspecific innate immune response to bacterial infection. Baldini et al. identified a C → T base change, 159 bases upstream of the transcription start site for CD14. They then studied children from the general population and showed the T genotype to be significantly associated with high levels sCD14 and low levels of IgE. Activation of monocytes and macrophages through CD14 causes the release of a complex mixture of cytokines which modulate the specific immune response and which have been shown to inhibit the production of IgE [292, 293].

Another potentially important candidate gene is the IL-4 receptor α (IL-4Rα) gene, located on chromosome 16q. One variant of the IL-4Rα gene (Gln576Arg) has been associated with increased signal transduction and high IgE levels, including the hyper-IgE syndrome [294].

The pro-inflammatory cytokine tumour necrosis factor (TNF) shows constitutional variation in the level of secretion which is linked to polymorphisms within the TNF gene complex and the surrounding MHC (located on chromosome 6p) [295]. TNF is prominent in asthmatic airways [296]. One study identified a higher incidence of asthma in subjects possessing allele 1 of the LTα *Nco*I polymorphism. However, linkage was not associated with IgE production, indicating that the association of the TNF polymorphisms with asthma is independent of atopy.

Chromosomes 13q and 12q have also been linked to asthma and related phenotypes but not the linked regions contain no known candidates [297].

Overall, a combination of genetic factors along with the environmental factors mentioned earlier, can contribute to the pathogenesis of allergic disease and predispose any given individual to allergy. Atopic diseases have increased in prevalence over the last few decades and now affect approximately 20-30% of the general population in developed countries. (see figure 1.2, pg 59).

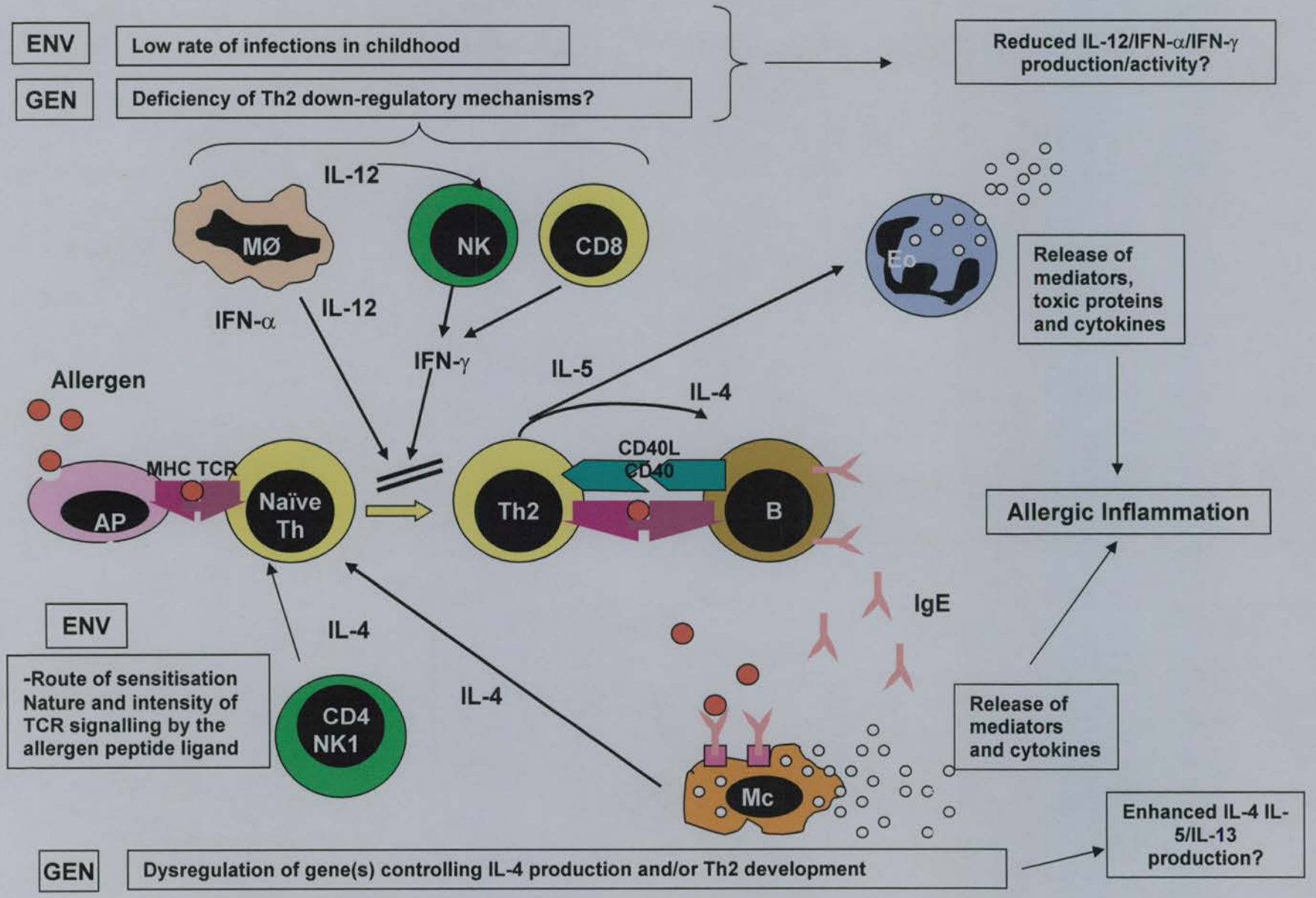
1.4.11 T Cell Receptor-α and Human Leukocyte Antigen-DR

Inhaled allergen sources such as house dust mite (HDM) or grass pollens are complex mixtures of proteins. Der p I and Der p II (major allergens from HDM) have four major B cell epitopes [298, 299] and peptide mapping of Der p II has shown that T cell clones from different individuals may also react to common T cell epitopes [300].

The human leukocyte antigen (HLA) and T cell receptor (TcR) genes are candidates for germ-line influences on specific allergen responses. The association of HLA haplotypes and ragweed allergy was the first human Ir (immune response) gene to be recognised [301], and HLA-DR restriction of IgE reactions to allergen is well documented [302, 303]. However, the HLA genes on their own do not account for the differences in an individual's IgE reactions to allergen [302]. Polymorphisms in the TcR genes influence the peripheral TcR repertoire [304, 305] and may affect the immune response to antigen. The Vα8 region of TcR-β has previously been demonstrated to be in excess in T cell clones reacting to HDM [306]. A later study showed that both Vα8.1*2 and HLA-DRB1*1501 were positively associated with IgE titres in Der p II [307]. The epitopes of Der p I recognised by human and mouse T cells will be discussed later in the chapter.

Figure 1.2 - The Pathogenesis of Allergy and Asthma

Adapted from Parronchi et al [168].



1.5 House Dust Mite Allergy

Approximately 20% of the developed world's population suffers from allergy to house dust mite (HDM). House dust mites are arthropods that measure between 170 – 350 μm in length and thrive in warm and humid conditions. In the United Kingdom, three species, *Dermatophagoides pteronyssinus, D. farinae* and *Euroglyphus maynei*, commonly flourish in the indoor environment. The mites produce faecal pellets that vary in size between 10 – 40 μm; this makes them ideal respirable vehicles for delivery to the respiratory tract. Der p 1 has been identified as being one of the major allergens found at high concentrations in mite faeces. It is highly water soluble with a molecular weight of 25 kD. It was found that approximately 75% of the serum IgE antibodies to *D. pteronyssinus* in sensitive patients were directed against Der p 1 [308].

Der p 1 has been cloned and sequenced and found to consist of 222 amino acids. It also has a high degree of homology at the nucleotide and amino acid level with actinidin and papain, which belong to the group of cysteine proteases [309]. The two crucial amino acid residues (cysteine and histidine) known to be an integral part of the active site of papain and actinidin are also conserved in Der p 1 (as cysteine residue 34 and histidine residue 170). As well as being an immunogenic protein in its own right, the protease activity of Der p 1 is also thought to contribute to the pathogenesis of airway inflammation. This enzymic activity is thought to cause disruption of intercellular tight junction (TJs), which are the principal components of the epithelial paracellular permeability barrier. Specifically, Der p 1 led to the cleavage of the TJ adhesion protein occludin in confluent airway epithelial cells resulting in increased epithelial permeability to a variety of allergens [310].

1.5.1 Cleavage of CD23 and CD25

A few years ago, two groups showed that Der p 1 could cleave CD23, the low affinity receptor for IgE, by proteolysis, [85, 86]. As mentioned elsewhere, cleavage of CD23 results in the formation of soluble CD23 (sCD23) molecules that retain their ability to bind IgE and thus exacerbate allergic symptoms.

More recently, Der p 1 has also been shown to proteolytically cleave the α subunit of the human IL-2 receptor (CD25) from peripheral blood T cells [87]. As mentioned before, IL-2 plays a pivotal role in the development of Th1 cells and thus a disruption in this signalling pathway would consequently bias the immune response towards Th2 induction.

1.5.2 Epitopes of Der p 1 Recognised by Human T Cells

The antigen and restriction specificity of T cell responses to Der p1 have been identified using cDNAs and various peptides. Five regions of the protein, residues 45-67, 94-104, 107-119, 110-131 and 117-143 have been identified that contain T cell epitopes restricted by several different HLA class II molecules [299]. T cell clones specific for residues 45-67 and 117-143 recognised antigen in association with HLA-DR7, whilst those T cells reactive with amino acids 94-104 were restricted by HLA-DR2, -DRw11 (DR5) and -DR8 [311]. Within the region 101-131 of Der p1, three overlapping epitopes are present that are similar to those determined in H-2^b mice. The T cell epitopes 110-131 and 110-119 are restricted by DRB1*0101 and DPB1*0402, respectively. The T cell epitope 107-119 was HLA-DP restricted and could be presented to T cells bound to HLA-DPB1*0401, -DPB1*0402 and – DPB1*0501-bearing accessory cells.

1.5.3 Epitopes of Der p 1 Recognised by Murine T Cells

When primed with Der p 1, mice of the H-2^b haplotype (e.g. C57BL/6, BALB/b) were found to be the highest responders. H-2^k mice had intermediate responses whilst H-2^d, H-2^g and H-2^k mice responded poorly (Hoyne - PhD. thesis). The murine T-cell epitopes have been identified using overlapping synthetic peptides. H-2^b mice recognise three distinct epitopes at residues 21-49, 78-100 and 110-131. The strongest response was observed to a 110-131 peptide which contains both a $CD4^+$ and $CD8^+$ epitope:

$CD4^+$	^{113}ISNYCQIYPPNANKI^{127}
$CD8^+$	^{111}FGISNYCQI^{119}

1.6 Immunotherapeutic Strategies for Atopic Diseases

With such a high incidence of atopic disorders in the developed world, it is clear that suitable therapies are needed. This can be approached in a number of ways. Corticosteroids are a commonly used anti-inflammatory which require continuous administration to be effective in reducing symptoms, are associated with undesirable side effects and most importantly, have no effect on the underlying inflammatory process. Therefore, it is preferable to target the allergen-specific T cells which are central to the induction and regulation of allergic responses. Allergen-specific T cells can be targeted either directly or indirectly. Non-allergen-specific immunotherapy can also be induced by antagonising the production of Th2 cytokines or by the use of anti-IgE therapies.

1.6.1 Allergen-Specific Immunotherapies

1.6.1.1 Tolerance

T cells can be functionally inactivated or tolerised by a number of different mechanisms, including clonal deletion, the induction of anergy (which occurs when antigen binds to the TCR but the T cells no longer proliferate) or the induction of linked suppression.

1.6.1.2 Anergy

Anergy was first defined *in vitro* as a state of functional unresponsiveness. It occurs when antigen binds to the TCR in the absence of adequate costimulation, mediated through the interaction of CD28 and its ligands, CD80 and CD86. This has been demonstrated by *in vitro* experiments, firstly, by incubating influenza virus-specific human T cell clones with the relevant virus-specific haemagglutinin peptide alone, in the absence of APC [312]. Treatment resulted in an inability to respond to rechallenge with antigen 24 hours later. A second study by Higgins at al. investigated the effect of an epitope located in the group 1 allergen of Der p on HLA-DP-restricted T cell clones. Pretreatment of the T cell clones with the Der p 1 peptide p(101-119) rendered them nonresponsive to an immunogenic challenge with the whole *D. pteronyssinus* allergen [311].

In vivo studies in mice have subsequently been developed and have shown that T cell responses to allergens can be inhibited by the subcutaneous, intranasal or oral administration of allergen-derived synthetic peptides. One murine study utilised peptides from the cat allergen Fel d 1. Mice were pre-treated subcutaneously with high dose peptide prior to a parenteral challenge with the same peptide in Freund's complete adjuvant. This resulted in the tolerisation of T cells, as evidenced by decreased production of IL-2, IL-4 and IFN-γ from lymph node and spleen cells cultured with the peptide. In addition T cell proliferation was decreased in the peptide-tolerised animals [313]. A later study by Hoyne et al. administered Der p 1 peptide intranasally. Four days after the peptide treatment, rapid but transient activation of MHC class II-restricted $CD4^+$ T cells was observed (by upregulation of the activation molecules CD25) however, by day 14, IL-2 and IFN-γ secretion by T cells were down-regulated as well as proliferation [314].

This so-called peptide treatment has been used in humans as a possible means of therapy. Peptide therapy has a considerable safety advantage over traditional therapies with crude extract or native allergens. Because the peptides are smaller than the whole allergen, there is reduced IgE cross-linking by allergen-specific peptides, with the desired effects on T cells unaltered [315]. Disadvantages of this approach to immunotherapy include the fact that large amounts of IL-4 and IL-5 are still produced during initiation of a peptide vaccine, that could lead to initial worsening of symptoms. In addition, most allergens contain multiple T cell epitopes that are restricted by different MHC class II antigens. Thus, the total T cell response to the native protein is limited to a few immunodominant epitopes but tolerance induction is avoided because non-immunodominant epitopes have made themselves available after *in vivo* processing of native allergen [316].

The modulation of proliferative and cytokine responses has been demonstrated following peptide immunotherapy in bee venom-allergic subjects [317]. Proliferative and cytokine Th1 and Th2 responses from isolated peripheral blood mononuclear cells were suppressed after bee venom peptide immunotherapy. Following whole allergen challenge, there was a change in the PLA_2-specific IgE:IgG4 ratio in favour of IgG4. However, the number of subjects in the experiment was small and comparisons were not made in relation to a placebo group. Akidis et

al. also found a reduction in T cell proliferative responses to allergen after ultra rush bee venom immunotherapy [318]. However, responses could be restored following addition of exogenous IL-2 or IL-15, which suggested that the T cells were in a state of anergy. Even when proliferation was restored, the anergy effect resulted in the production of a stronger Th1 phenotype.

The precise mechanisms underlying the induction of anergy still need to be fully elucidated. However, it is known that peptide-mediated anergy is associated with compound changes in the T cell phenotype characterised by the down-regulation of the TCR/CD3 receptor complex and enhanced expression of CD2 and CD25 [319, 320]. Anergic cells have an inability to release intracellular calcium upon restimulation; and altered signalling pathways may be utilised by anergic T cells [321, 322]. Studies of peptide anergised PLA_2-specific cloned $CD4^+$ T cells have identified abrogation of the activity of p56lck and ZAP-70 tyrosine kinases [323].

Expression of the costimulatory molecules CD80 and CD86 does not seem to affect the induction of anergy. No changes in their expression were observed on Th0 or Th2 cells that had become unresponsive after modulation. Also, in the absence of APCs, stimulation with peptide, induced anergy in human Th2 cells irrespective of CD80 and CD86 [320]. However, CD28 expression may be down-regulated [324].

1.6.1.3 Clonal Deletion

In the periphery, a mature T cell will be stimulated to proliferate and produce effector cells if it encounters an antigen for which its receptor is specific. However, when a developing T cell encounters the antigen it recognises in the thymus, it undergoes apoptosis. This is the basis of negative selection and has been demonstrated in experiments involving transgenic mice. High dose antigen treatment in OVA-TCR transgenic mice, induced clonal deletion by apoptosis [325]. Self peptides bound to self MHC molecules on thymic cells and developing cells with receptors specific for these peptides, will be deleted from the repertoire. This form of tolerance is incredibly important because it prevents the body reacting to self antigens. Breakdown in clonal deletion leads to autoimmune disease.

Cytotoxic T Lymphocyte-Associated Antigen 4 (CTLA-4) plays a key role in immunologic self-tolerance in the periphery. CTLA-4 (CD152) is a CD28 homologue expressed on activated T cells, and upon interaction with CD80 or CD86 on APCs, exerts a downregulatory or attenuating effect on T cell-mediated immune responses [326, 327]. CTLA-4-deficient mice develop a severe lymphoproliferative disorder and die from autoimmune-like disease within one month after birth [52, 328]. In the murine models of multiple sclerosis and insulin-dependent diabetes mellitus, the autoimmune responses were exacerbated after administration of anti-CTLA-4 antibody [329, 330]. Perez et al. demonstrated the requirement of CTLA-4 for in-vivo peripheral tolerance induction [331].

1.6.1.4 Linked Suppression and Regulatory T Cells

In addition to clonal anergy or deletion, there is accumulating evidence to suggest that another tolerance mechanism exists involving T cell mediation, termed linked suppression. A phenotypically and functionally distinct population of T cells has been identified and termed regulatory T (T reg) cells. This subset of cells were implicated in the control of intestinal inflammation and have been suggested to play a pivotal role in intestinal homeostasis [332]. These cells express a particular cell surface phenotype ($CD25^{+}CD45RB^{lo}CD4^{+}$), determined through studies investigating inflammatory bowel disease [333] and autoimmune gastritis [334, 335]. Colitis induced in SCID mice by the transfer of $CD45Rb^{hi}CD4^{+}$ T cells was prevented by the co-transfer of murine Tr1 clones derived from $CD4^{+}$ T cells expressing a transgenic TCR specific for OVA. Immunosuppression was dependent on the antigen-induced activation of Tr1 cells *in vivo* as these cells only inhibited colitis in recipients receiving OVA in their drinking water. In contrast, the effector function of Tr1 cells was not antigen-specific as they could inhibit the function of T cells responding to unknown intestinal antigens, a phenomenon termed antigen-driven bystander suppression (Groux, O'Garra et al. 1997).

In the colitis model, immune suppression was dependent on TGF-β [336] and production of IL-10 [337], along with the ligation of CTLA-4 [338]. The role of CTLA-4 in immune suppression was also shown by Takahashi et al. by blocking CTLA-4 *in vivo* [339]. CTLA-4 has a 10 to 100-fold higher affinity for CD28 than

B7.1/2. The authors suggested that T cells stimulated via CTLA-4 and the TCR may physically interfere with the interaction of other T cells with APCs by competing for the costimulatory molecules on the APCs, as they are predominantly expressing CTLA-4 [340]. Alternatively, the signals via the TCR and CTLA-4 may activate $CD25^{+}CD4^{+}$ T cells to deliver to the other T cells a negative signal for activation and proliferation. This is currently under investigation.

There is evidence of a role of cell-cell contact in bystander suppression. Hoyne et al. have proposed a mechanism in which the differentiation of $CD4^{+}$ T cells into regulatory phenotypes, may be induced by the interaction of two receptors – Notch-1 (expressed on T cells) and Serrate-1 (expressed by APCs), that also determine cell fates during thymic T-cell differentiation [341]. The expression and function of Delta-1 (a second ligand for Notch), was also investigated in a murine model of T cell tolerance to Der p 1 *in vivo* by using *in-situ* hybridisation and virus-mediated gene transfer [342]. The intranasal delivery of high dose peptide induced the transient expression of Delta-1 on inhibitory $CD4^{+}$ T cells. Ligation of the Notch-1 receptor on neighbouring T cells by Delta-1^{+} regulatory T cells inhibited clonal expansion thus implicating Notch/Delta signalling as an important factor in the underlying mechanisms of linked suppression.

1.6.1.5 Redirection of Allergen-Specific Th2 Responses

As mentioned earlier, the Th2 response is quite resistant to a reversal in cytokine patterns once established; and after long-term stimulation, this reversibility is completely lost. However, recent findings indicate that regardless of prior commitment, memory $CD4^{+}$ T cells may retain the capacity to be further influenced during effector cell development [343, 344].

1.6.1.6 Altered Peptide Ligands

Several studies suggest that certain epitopes can preferentially induce one of the Th subsets. The chosen response can be induced by altering either the antigenic peptide or the MHC class II molecule. High MHC class II peptide density on the APC surface favours Th1 responses, while lower ligand densities favour Th2 responses [47]. This is logical as allergens are presented to the human immune system at very low doses, leading to IgE synthesis. Moreover, desensitisation is

generated by injecting successively higher doses of allergen. Kumar et al. showed that stimulation with a high affinity ligand resulted in IFN-γ production whilst low affinity ligands induced IL-4 production [345]. A single MHC polymorphism may dictate Th1/Th2 selection by determining the level of peptide presented to a given TCR on APCs [346].

Slightly later studies showed the enhancement of IFN-γ production from human Th0 cells by stimulation with an altered dominant T cell epitope of *Der p 2* [347]; and similarly, a replacement of the arginine residue at position 21 by lysine in a peptide derived from the *D. farinae* group 1 allergen, resulted in increased IFN-γ production associated with a rise in IL-12 [348].

More recently Janssen et al. defined four modulatory peptide analogues of ovalbumin ($OVA_{323-339}$) with comparable MHC class II binding affinity. These peptide analogues were used for immunotherapy by subcutaneous injection in OVA-sensitised mice before OVA challenge. Treatment with a Th2-skewing peptide dramatically increased airway eosinophilia upon OVA challenge but treatment with a Th1-skewing peptide analogue resulted in a significant decrease in airway eosinophilia and OVA-specific IL-4 and IL-5 production [136].

1.6.1.7 Use of Adjuvants and Incorporation of Peptides

Peptides and proteins can be administered along with adjuvants that preferentially induce a Th1 response. Examples are the use of recombinant *M. bovis* (BCG) against *L. major* [349], *M. tuberculosis* [350] and measles virus [351]. Live recombinant Salmonella has been administered orally to elicit antigen-specific Th1-type responses in both mucosal and systemic tissues, in the absence of IL-4 and IL-5. In the laboratory, a common adjuvant administered in mice to induce a Th1 response, is Complete Freund's Adjuvant. However, because of its undesirable inflammatory side effects, CFA is not suited for use in human vaccines. The main component of this adjuvant are mycobacterial cell walls that induce high IL-12 production by macrophages, with subsequent upregulation of IFN-γ [352]. In contrast, Incomplete Freund's Adjuvant, alum, acellular Bordetella pertussis toxin and cholera toxin act by inducing Th2 responses [353-355].

Immune stimulating complexes (ISCOMS) act as antigen carriers. They are generally 40 nm in diameter and consist of cholesterol, phospholipids, the chosen antigen and the adjuvant component, *Quillaja* saponins. Studies with ISCOMS have shown that they can upregulate both Th1 and Th2 responses [554-556]. As well as this, ISCOMS have also been shown to induce $CD8^+$ cytotoxic T cells, when gp160 envelope glycoprotein from HIV was incorporated and given subcutaneously in mice [356].

Other antigen carriers include liposomes and MEA (microencapsulated antigen) particles which will be discussed in more detail later in this thesis. Liposomes can simply be portrayed as spherical vesicles consisting of one or more phospholipid bilayers surrounding an aqueous cavity. As well as drug delivery and gene therapy [357, 358], liposomes can be employed as an adjuvant to enhance the immunogenicity of small antigens [359, 360].

1.6.1.8 Plasmid DNA Gene Therapy

Plasmid DNA vaccines usually consist of naked DNA (i.e. DNA that has been freed of all the proteins in the usual DNA-protein complexes) coding for the gene of interest inserted into a plasmid. Plasmids are circular DNA molecules, usually found in bacteria and some yeasts. Although plasmids are not usually essential for cell growth and division, they often confer traits (e.g. antibiotic resistance) on the host organism, which can be a selective advantage under certain conditions. A plasmid can reproduce itself in bacteria but not in other cell types unless the appropriate transcriptional elements are present [361]. When DNA encoding an antigen is injected intramuscularly or intradermally, host cells take up the foreign DNA, express the gene of interest and synthesise the corresponding protein. The protein then enters the MHC class I pathway, where subsequent peptide fragments of the protein are carried to the cell surface and cell-mediated immunity in the form of cytotoxic $CD8^+$ T cells is induced, against later rechallenge with whole protein [362].

DNA vaccines have many advantages over standard vaccines. The immunity they induce is long lasting [363], they can induce the expression of antigens that

resemble native epitopes more closely because the manufacture of live attenuated vaccines and killed vaccines often alters their structure and thus lowers the antigenicity.

Gene therapy has been investigated quite extensively over the past ten years. One of the first reports of gene therapy was by Wolff and colleagues in 1990. They reported that non-replicating DNA plasmids encoding the reporter genes chloramphenicol acyltransferase, luciferase and β-galactosidase, could be internalised and expressed in muscle cells following i.m. injection without the need of any transfection vehicle [364]. Since then, gene therapy has been used in a number of animal disease models, for example, HIV, HSV and *Leishmania major* infection [365-367].

Gene therapy is now being investigated as an approach for the treatment of allergic disorders. An initial study in 1996, showed that mucosal IFN-γ gene transfer (by intratracheal instillation) resulted in significant expression of IFN-γ in the pulmonary epithelium, as well as an inhibition in both Ag- and Th2-cell-induced pulmonary eosinophilia and airway hyperreactivity [368]. A second study in the same year reported an inhibition of specific IgE to Der p 5 allergen after intramuscular injection of pDNA encoding Der p 5 in mice. Furthermore, this suppression of the IgE response could be adoptively transferred with $CD8^+$ cells to naïve and subsequently rechallenged recipients, suggesting that active suppression of this IgE response was involved [369]. At around the same time, it was found that certain bacterial DNA motifs were able to favour Th1 responses [370, 371]. This motif – an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines, rapidly stimulated B cells to produce IL-6 and IL-12, $CD4^+$ T cells to produce IL-6 and IFN-γ, and NK cells to produce IFN-γ both *in vivo* and *in vitro* [370]. A study in 1998 by Broide et al, demonstrated that immunostimulatory sequences (ISS) containing a CpG DNA motif, significantly inhibited airway eosinophilia and reduced responsiveness to inhaled metacholine [372]. A single dose of ISS was found to inhibit eosinophilia as effectively as seven daily injections of corticosteroids. Moreover, whilst IL-5 levels were reduced with both ISS and steroid treatment, only the ISS could induce allergen-specific IFN-γ production and redirect the immune response toward a Th1 type.

More recently, Hertz and colleagues delivered a DNA vaccine encoding murine IL-5 modified to contain a promiscuous foreign Th epitope to break or bypass immunological tolerance to IL-5. In established models of experimental asthma, Th-modified IL-5-vaccinated mice induced an immune response directed against IL-5 that reduced both pulmonary lymphocyte infiltration and eosinophilia, reduced the levels of Th2 cytokines, and inhibited the development of AHR [577].

CpG-DNA acts as a "pathogen-associated" molecular pattern that is recognised by TLR-9 (Toll-like Receptor 9) [575]. Dendritic cells are major expressors of TLR-9. Interactions between CpG-DNA and TLR-9 rapidly activate antigen-presenting dendritic cells through the ancient Toll/IL-1-receptor signalling pathway to upregulate costimulatory molecules and to produce Th1-polarising cytokines, such as IL-12 and IL-18. Thus, interactions between CpG-DNA and TLR-9 effectively bridge innate and acquired immunity [576].

Some concerns exist however, over the safety of DNA vaccines. One major factor is whether plasmid DNA can insert itself into the host genome; if so, then insertional mutagenesis would be a concern. There is evidence against integration of the DNA into the host chromosome [363], although it is difficult to prove conclusively that integration cannot happen. The evidence to date suggests that increased pDNA antigen expression occurs for a limited time period so, how would this affect the immune system? It may lead to host tolerance or an attack on tissues expressing that antigen but this has yet to be proven [168].

1.6.2 Non-Allergen-Specific Immunotherapies

It has been proven in IL-4-deficient mice that Th2 responses are probably not critical for survival and protection [373], however, it has been shown that a Th2 environment is critical for foetal survival in pregnancy [88]. In addition, Th2 responses play an important part in the balance between developing allergic or autoimmune responses. Therapies that target the Th2 pathway have been developed even though they may be detrimental to health.

It has been mentioned earlier, that transcription factors such as the c-maf oncogene are selectively transcribed; and signalling by IL-4 through STAT6 occur in Th2 cells. This makes them likely candidates for manipulating Th2 responses.

Shimoda and Takeda have shown that knock out of the STAT6 gene results in deficient Th2 responses [112, 113].

The Th2 cytokines IL-4 and IL-5 can be antagonised by soluble receptors [374], antibodies [375, 376], mutant proteins [377] and even by a specific gene transcription inhibitor [378]. Renz et al used lymphocytes from allergen-sensitised mice and restimulated them *in vitro* with the sensitising allergen in the presence of either a soluble murine sIL-4R, a dimeric sIL-4Rig fusion protein (sIL-4R/Fc) or anti-IL-4 antibody. Both the monomeric and dimeric sIL-4R inhibited allergen-specific IgE and IgG1 similar to that observed in the presence of the anti-IL-4 antibody. In kinetic experiments, Renz found that newly induced IgE production was the major target of the sIL-4Rs, demonstrating the efficacy of sIL-4R in inhibiting the early stages of the IgE B cell maturation pathway [374].

Two humanised monoclonal antibodies specific for IL-5 have been investigated as potential therapies for asthma. SCH55700 (reslizumab) is a humanised monoclonal antibody with activity against IL-5 from various species [578]. SB240563 9 (mepolizumab) is also a humanised antibody with specificity for human and primate IL-5 [579, 580].

In a dose-dependent manner, SCH55700 inhibited total cell and eosinophil influx into BAL fluid, bronchi, and bronchioles of allergic mice for up to 8 weeks after a single 10 mg/kg dose and for 4 weeks after a single 2 mg/kg dose. In allergic guinea pigs, SCH55700 caused a dose-dependent decrease in pulmonary eosinophilia and inhibited the development of allergen-induced airway hyperresponsiveness to substance P. it also inhibited the accumulation of total cells, eosinophils, and neutrophils in the lungs of guinea pigs exposed to human IL-5. SCH55700 had no effect on the numbers of inflammatory cells in unchallenged animals or in animals challenged with GM-CSF, and had no effect on the levels of circulating total leukocytes [578]. A rising single dose phase I clinical trial was conducted with SCH55700 in patients with severe persistent asthma who remained symptomatic despite intervention with high-dose inhaled or oral steroids. The two highest doses of SCH55700 significantly decreased peripheral blood eosinophils, with inhibition lasting up to 90 days after the 1 mg/kg dose. There was also a trend toward improvement in lung function at the higher doses 30 days after dosing, with mean

FEV_1 increasing by 11.2 and 8.6% in the 0.3 and 1.0 mg/kg groups, respectively, versus 4.0% in the placebo group [581].

Preclinical studies with SB240563 in cynomolgus monkeys indicated that peripheral blood eosinophils were decreased as a result administration of the antibody [579, 580]. SB240563 has also been tested in asthmatic persons in a clinical single dose safety and activity study [582]. Patients with mild asthma were administered with a single intravenous dose of the antibody at either 2.5 or 10 mg/kg, or placebo. Patients were challenged with allergen 2 weeks before and 1 and 4 weeks after dosing. Peripheral blood and sputum eosinophil levels were measured, and early-phase and late-phase asthmatic responses were assessed by measuring the percentage fall in FEV_1 induced by allergen challenge. Both doses of SB240563 caused a significant reduction in peripheral blood eosinophils. Eosinophil counts were reduced in the 10 mg/kg dose group by approximately 65% for up to 8 weeks. Post challenge sputum eosinophils were also reduced in the 10 mg/kg dose group.

In a rat model of allergic lung inflammation, eosinophil influx into the lung was significantly diminished by the use of monoclonal antibodies against various integrins [379]. Anti-CD4 monoclonal antibody therapy has also been demonstrated as a therapy for rheumatoid arthritis and psoriasis [380] as well as in transplantation models [381].

IgE antibodies can also be targeted. In recent years, anti-human IgE antibodies have been developed that bind to membrane IgE on B cells and soluble IgE but not with basophil or mast cell bound IgE [382, 383]. In another study, IgE antibody treatment not only neutralised serum IgE but also inhibited eosinophil recruitment in Der p 1-sensitised mice and the production of IL-4 and IL-5, by inhibiting IgE-CD23-facilitated presentation to T cells [384]. Studies using anti-IgE antibodies in asthmatic patients have been shown to be effective in reducing serum IgE and look promising for the future [385, 386].

1.7 Mucosal Immunity

1.7.1 Antigen Uptake and Processing in the Gut Associated Lymphoid Tissue (GALT)

With an area of over 300 m^2, the gastrointestinal tract is the largest mucosal surface in man. The vast majority of cells comprising the intestinal epithelium are absorptive enterocytes that are designed to absorb nutrients, but to exclude foreign macromolecules and microorganisms. Enterocyte apical surfaces are coated by rigid, closely packed microvilli [387] that are coated by the filamentous brush border glycocalyx, a thick (400-500 nm) layer of membrane-anchored glycoproteins [388]. The glycocalyx is an effective diffusion barrier that contains large, negatively charged integral membrane mucin-like molecules, [388], absorbed pancreatic enzymes and stalked intramembrane glycoprotein enzymes responsible for terminal digestion [389]. This thick, highly glycosylated layer prevents direct contact of most macromolecular aggregates, particles, viruses and bacteria with the microvillous membrane [390]. Thus, the glycocalyx serves two complimentary roles – protection against invading pathogens whilst providing a highly degradative microenvironment to promote the digestion and absorption of nutrients.

The GALT can be divided into a number of layers [391, review], the first of which consists of intraepithelial lymphocytes (IELs) which reside within the epithelium itself, above the basement membrane. The IEL population chiefly consists of $CD8^+$ T cells, with $CD4^+$ T cells being the predominant LPL population. Beneath this layer, in the lamina propria located between the epithelium and submucosa, reside the lamina propria lymphocytes (LPLs). Finally, organised lymphoid tissue in the form of Peyer's Patches are present within the gut epithelium in the small intestine [392].

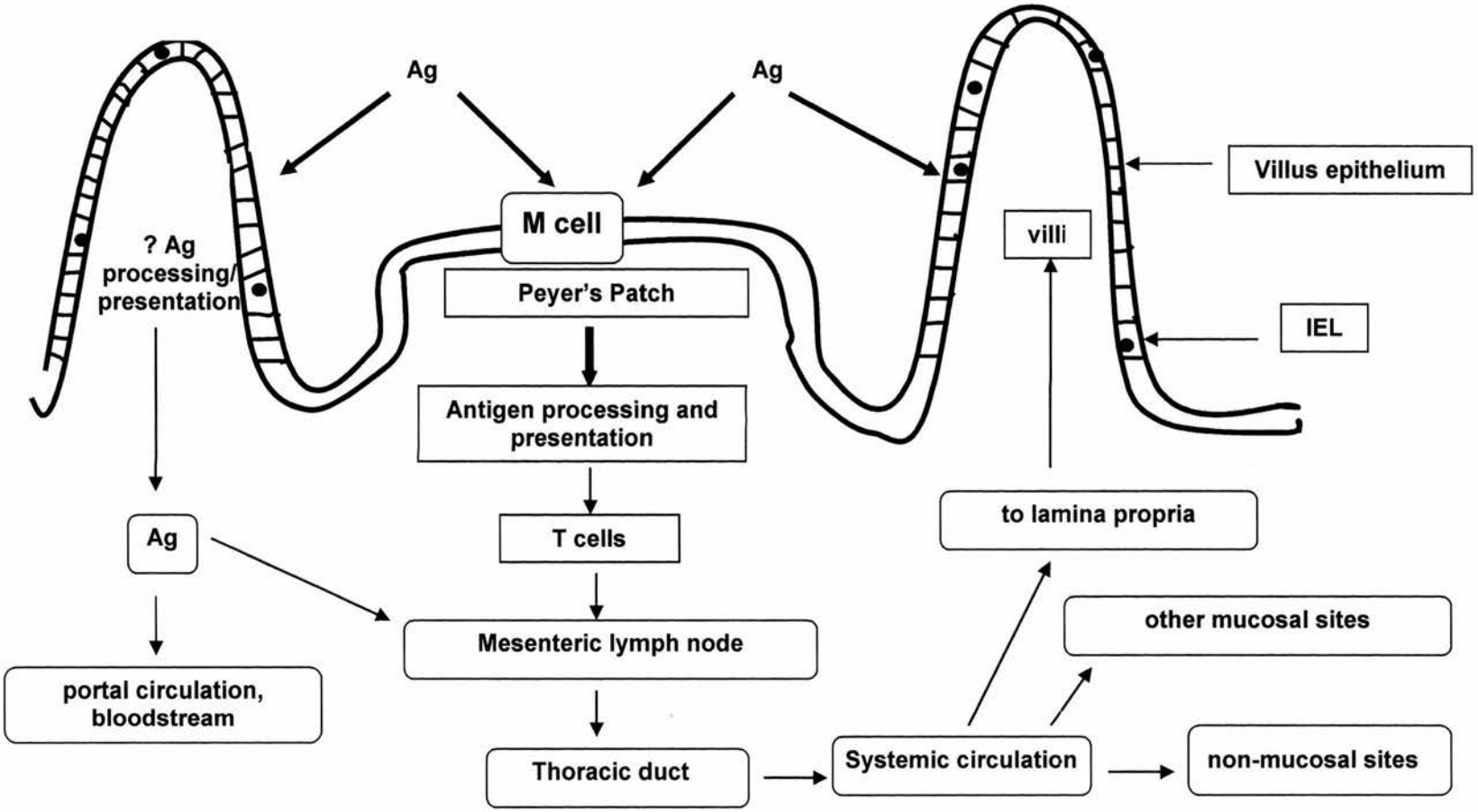
The Peyer's Patches have a specialised epithelium known as M cells, which deliver samples of foreign material by vesicular transport from the lumen directly to intra-epithelial lymphoid cells and to organised mucosal lymphoid tissues. Most M cells lack the thick glycocalyx seen in other enterocytes and therefore, in its absence, uptake of foreign materials is improved [393]. Hydrophobic particles and cationic macromolecules adhere much more avidly to M cells than to enterocytes. Several

investigators have observed M cell-selective adherence and uptake of polystyrene or latex beads [394], PLG microparticles [395] and liposomes [396, 397]. Antigen is endocytosed by the M cell on the apical surface, transcytosed to the basolateral side by vesicular transport and presented to the underlying follicle-associated epithelium. Below the FAE lies an extensive network of dendritic cells and macrophages intermingled with $CD4^+$ T cells and B cells from the underlying follicle [398]. These subepithelial cell populations reinforce the idea that M cells serve as gateways to immune inductive sites, where endocytosis and killing of incoming pathogens as well as processing, presentation and perhaps storage of antigen occurs. The APCs present the antigen along with MHC class II to T cells, which subsequently migrate via the efferent lymphatics along with IgA-committed B cells sensitised to the antigen, through the mesenteric lymph nodes to the thoracic duct and hence into the circulating blood to other parts of the body (mucosal and non-mucosal), (see figure 1.3 pg 76).

The cytokine milieu in the lymphoid tissue microenvironment strongly influences the generation of T cells which provide help to induce and differentiate IgA^+ B cells [399]. Mature (antigen responsive) B lymphocytes expressing membrane IgM switch to surface IgA production, under the control of Th2 cytokines including TGF-β, IL-4, IL-5 and IL-10 [400]. This switching occurs in the germinal centres of the organised mucosa-associated lymphoid tissues (*O*-MALT). When IgA^+ cells leave the *O*-MALT they migrate to distant mucosal sites to undergo terminal differentiation from soluble IgA^+ B cells to IgA-secreting plasma cells under the regulation of IL-5 and IL-6 [401, 402]. The IgA antibodies are secreted as dimers associated with a single J chain which then diffuses across the basement membrane and is subsequently bound by the poly-Ig receptor (pIgR) on the basolateral surface of the epithelial cell. The bound complex undergoes transcytosis in which it is transported in a vesicle through the cell to the apical surface. Here, the pIgR is cleaved enzymatically, releasing the extracellular portion of the receptor still attached to the Fc region of the dimeric IgA. This fragment of the receptor, called the secretory component, is thought to help protect the dimeric IgA from proteolytic cleavage [403]. IgA antibodies are important in preventing the attachment of bacteria or toxins to epithelial cells or the absorption of foreign substances, and provide a first

line of defence against a variety of pathogens. In fact, 70-90% of all Ig secreted in the intestinal mucosa is IgA [392].

Figure 1.3 - Antigen and T Lymphocyte Traffic in the Gut Associated Lymphoid Tissue



1.7.2 Mucosal Tolerance

As discussed earlier, immunologic tolerance provides the immune system with self-nonself discrimination and is crucial to prevent pathogenic reactivities against self-antigens. Most of the population have lifelong clinical and immunological tolerance to both food antigens and their gut flora. However, a sizeable minority of the population (5%) suffer from adverse reactions to foods, of which 40% are thought to have a known immunological basis. Food-allergic diseases are a feature of childhood and may represent a breakdown or failure of oral tolerance induction or maintenance.

Oral exposure to antigens has several potential outcomes:

1) induction of systemic immunological hyporesponsiveness (tolerance),
2) systemic priming, and/or
3) the induction of local secretory IgA responses in the absence of measurable systemic immune responses.

The oral administration of antigens is a classical method of inducing antigen-specific peripheral immune tolerance [404]. The mechanisms of oral tolerance include active suppression, clonal anergy and clonal deletion. The dose of antigen, duration, and route of antigen administration seem to be crucial factors for determining which of these mechanisms will be the main operator [see 557 & 558 for reviews]. Orally administered antigens rapidly gain access to mucosal and systemic lymphoid tissues, and regulatory cells have been demonstrated in the Peyer's patches and mesenteric lymph nodes within 24 hours after a single feeding of antigen [405], and in the spleen after 4-7 days. Oral tolerance to systemic challenge is well established within 5-7 days [404, 406], and recent work suggests that antigen-specific suppression after a feed is demonstrable at 24-48 hours (Strobel and Mowat unpublished). Thereafter, tolerance seems to be long-lasting, with suppression of DTH being demonstrable for up to 17 months after a single feed [407], although the accompanying suppression of antibody responses is more short-lived and is lost after 3-6 months.

Friedman et al. were some of the first investigators to delineate the effect of antigen dose on the induction of tolerance mediated by either anergy or active suppression [408]. In this study, the investigators fed hen egg white lysozyme to mice and guinea pigs, and myelin basic protein to rats using low dose or high dose

regimens. A single feeding of high dose antigen (5-20 mg or 0.5 mg g^{-1} body weight in mice) induced tolerance characterised by anergy with little or no active suppression and increased secretion of IL-4. The most important change in anergic T cells is their inability to produce IL-2: this prevents them from proliferating and differentiating into effector cells when they encounter antigen, even if the antigen is subsequently presented by professional APCs. The anergic state can be reversed if the cells are cultured in IL-2. Anergy was confirmed by an increase in the frequency of IL-2-secreting cells following culture in recombinant IL-2. In contrast, a low dose (1mg or 0.1mg g^{-1} body weight in mice) intermittent feeding schedule, induced tolerance characterised by antigen-driven active suppression with increased secretion of transforming growth factor β (TGF-β) and IL-4 with minimal anergy. The *in vivo* relevance of bystander suppression was confirmed by the demonstration that injection of anti-TGF-β mAb into animals reversed oral tolerance induced by low-dose antigen [409]. In addition, feeding myelin basic protein (MBP) suppressed experimental autoimmune encephalomyelitis (EAE) induced not only by MBP but also by proteolipid protein (PLP) [410].

TGF-β is a potent immunosuppressive and anti-inflammatory cytokine, which plays an important role in the local function of the gut, as it serves as a switch factor for IgA production in the mucosa [411] and may also be involved in the homing mechanism of cells to high endothelial venules [412]. TGF-β is produced by both $CD4^+$ and $CD8^+$ GALT-derived T cells [413, 414] and is an important mediator of the active suppression component of oral tolerance. The importance of TGF-β in the maintenance of immune homeostasis is demonstrated in TGF-β-deficient mice, which have inflammation affecting multiple organ systems [415]. Chen et al. isolated T cell clones from the mesenteric lymph nodes of SJL mice that had been orally tolerised to myelin basic protein [413]. These clones were $CD4^+$ and were structurally identical to Th1 encephalitogenic $CD4^+$ clones in T cell receptor usage, MHC restriction and epitope recognition. However, they produced TGF-β with various amounts of IL-4 and IL-10 and suppressed EAE induced with either MBP or proteolipid protein. Thus, there appears to be a unique $CD4^+$ T-cell subset that induces mucosal helper T-cell function and downregulatory properties for Th1 and other immune cells. In contrast to Th1 and Th2 cells, these cells (termed Th3 or Tr -

T regulatory cells) provide help for IgA production and primarily secrete TGF-β. Th3/Tr cells appear distinct from Th2 cells since $CD4^+$ TGF-β-secreting cells that suppress a form of collitis have been generated from IL-4-deficient animals [336].

A regulatory population of T cells has also been identified in a nasal tolerance induction model. Shi et al. studied nasal tolerance induction as a strategy to prevent Experimental Autoimmune Myasthenia Gravis (EAMG) in Lewis rats and found that acetylcholine receptor (AchR) administration by the nasal route was as effective as oral administration at only 1/500 of the dose [416]. A later study using the same model suggested that suppression was independent of $CD8^+$ and involved Ag-specific $CD4^+$ Th3 cells producing TGF-β.

It is now clear that oral tolerance can affect $CD8^+$ as well as $CD4^+$ T cell functions and can be adoptively transferred by both these subsets [417, 418]. A recent study by Grdic et al. addressed the role of $CD8^+$ T cells in oral tolerance and whether evidence for compartmentalisation of control of the tolerant state could be observed [419]. They found that feeding KLH antigen to mice induced tolerance that was highly compartmentalised, requiring $CD8^+$ T cells for local suppression of IgA responses, while $CD4^+$ T cells were responsible for systemic hyporesponsiveness with Th1 and Th2 cell function equally affected. These findings confirmed those of earlier study by Garside et al. who found that the production of Th1 and Th2-dependent cytokines were equally affected [420]. Garside also found that oral tolerance could be induced in $IL4^{-/-}$ mice suggesting that Th2 cells were not essential in the induction of oral tolerance. However, a subsequent study by the same group showed that high dose tolerance induction with ovalbumin was dependent on $CD4^+$ T cells, as depletion of this population completely abrogated the tolerance [421].

The induction of anergy in high dose oral tolerance specifically affects antigen-specific Th1 lymphocytes (characterised by diminished production of IgG2a, IL-2 and IFN-γ) with intact Th2 responses (IgG1 and IL-4) [422]. The anergic state is probably the result of aberrant presentation of antigen by APCs lacking costimulatory molecules such as B7.1/B7.2 and intercellular adhesion molecule (ICAM-1) [423].

There are several candidates for the tolerogenic APC. Dendritic cells are obvious candidates due to their abundance throughout the intestinal wall, Peyer's

Patches and draining lymphoid tissues [409, 414, 424]. Administration of the growth factor Flt3L (ligand of *fms*-like tyrosine kinase) to mice expands the number of DCs in the intestine and other lymphoid organs, and increases the susceptibility to tolerance induction by feeding OVA [425]. B cells are unlikely to be involved in oral tolerance as peripheral T cell tolerance can be induced in B-cell-knockout mice [426].

The lining of the intestinal epithelium consists of enterocytes which express low levels of MHC class II. Epithelial cells lines have been shown to be capable of processing and presenting protein antigen to $CD4^+$ T cell hybridomas *in vitro* [427]. In support of this, recent studies have shown that orally administered ovalbumin can be detected in murine enterocytes 5-10 minutes after a feed [428]. However, as enterocytes do not express ICAM-1 or B7.1 [423]. It seems likely that presentation of antigen by these cells would normally lead to anergy of naïve $CD4^+$ T cells.

In addition, studies have demonstrated that epithelial cells may present antigen to $CD8^+$ T cells in the context of the CD1 molecule, a nonclassical MHC that is only distantly related to the MHC class I molecules [429]. Furthermore, the membrane protein gp180 expressed on epithelial cells has been reported to act as a cofactor together with CD1 in stimulating $CD8^+$ T cell activation [430]. Thus, the surface expression of the CD8 molecule is critical for interactions between epithelial cells and gut T cells, in particular for the activation of a regulatory $CD8^+$ population. Therefore, in addition to Peyer's Patches [424], the gut epithelium is also a prime site for the induction of tolerance.

Clonal deletion is rarely found in peripheral tolerance to nominal antigens and has not been described during oral tolerance induction in normal animals. Clonal deletion can be induced in transgenic animals but unphysiologically large doses of antigen were required to demonstrate such effects [325].

So far, therapeutic applications of oral tolerance have mainly been concerned with the treatment of autoimmune diseases [reviewed in 557]. Therefore, it has been proposed that mucosal tolerance is superior in suppressing Th1-biased immune responses. As already discussed, this may be due to preferential activation of lymphocytes producing cytokines that can be attributed to Th2-like cells (eg. IL-4, IL-5 and IL-10), which down-regulate Th1-based inflammatory processes.

Although not as abundant as Th1 studies, a few studies on Th2 tolerance induction do exist. In 1998, Wu et al. investigated the effects of continuous low-dose oral administration of ovalbumin [431]. TCR-transgenic mice were immunised intra-peritoneally with OVA in alum and pertussis toxin. Seven days later the mice received 100μg OVA/day for fourteen days orally before receiving a second challenge of OVA in alum/pertussis toxin one day later. This immunisation protocol induced Ag-specific unresponsiveness of Th2 cells without affecting the Th1 response, which resulted in suppression of Ag-specific IgE production. It was found that oral treatment induced down-regulation of the expression of Ag-specific TcR as well as B7.2 expression on APCs but did not cause clonal deletion of the T cells. A recent study by Russo et al. exposed different strains of mice to 1% ovalbumin dissolved in drinking water for 5 consecutive days [432]. This regimen of OVA administration induced Ag-specific unresponsiveness in all the mouse strains tested, characterised by reduced (almost absent) airway eosinophilic inflammation, airway hyperreactivity and mucus production; there were also low levels of Th2-type cytokines in bronchoalveolar lavage fluid and decreased OVA-specific IgG1 and IgE production. Tolerance in this model was not associated with IL-10 and TGF-β production or with immune deviation toward the Th1 pathway. However, the tolerance was short-lived as challenge at day 14 after oral treatment increased allergic responses.

Experimental tolerance induction is usually induced in naïve lymphocytes. However, in the disease state the immune system has already met the causing antigen and accordingly has developed active immune responses. Hence, studies of tolerance induction in primed animals would be more clinically relevant. A few studies do exist that report the effect of antigen feeding after antigen immunisation with mixed results. For example, feeding low doses of antigen after immunisation led to worsening of experimental autoimmune encephalitis [433] and similarly, was not effective in decreasing ongoing clinical EAE [434]. In contrast, feeding antigen seven days after immunisation produced dose-dependent suppression of T cell responses, although serum IgG levels were unaffected [435]. A recent study by Chung et al. investigated the effect of single or multiple feeding regimens on the induction of Ag-specific unresponsiveness in primed animals [436]. The results of

this study indicated that the early phases of the immune response could be tolerised by orally administered antigen. However, once memory was established to a particular antigen, feeding the same antigen was not effective in inducing tolerance.

On a similar theme, Hurst et al. investigated the effects of ongoing Th1 and Th2 responses during the establishment of aerosol-induced IgE tolerance [437]. They found that concurrent, secondary Th2 lung responses to keyhole limpet hemocyanin or primary responses to *Nippostrongylus larvae* or *Asperigillus fumagatus* extract prevented establishment of IgE tolerance to aerosolised OVA. Intranasal rIL-4 given before OVA aerosolisation also prevented establishment of tolerance, whereas concurrent Th1 responses to influenza virus or *Mycobacterium bovis* BCG had no effect. These results suggested that normal IgE and IL-4 unresponsiveness following Ag inhalation may be converted into sensitisation in the milieu of a concurrent, IL-4 producing Th2 response.

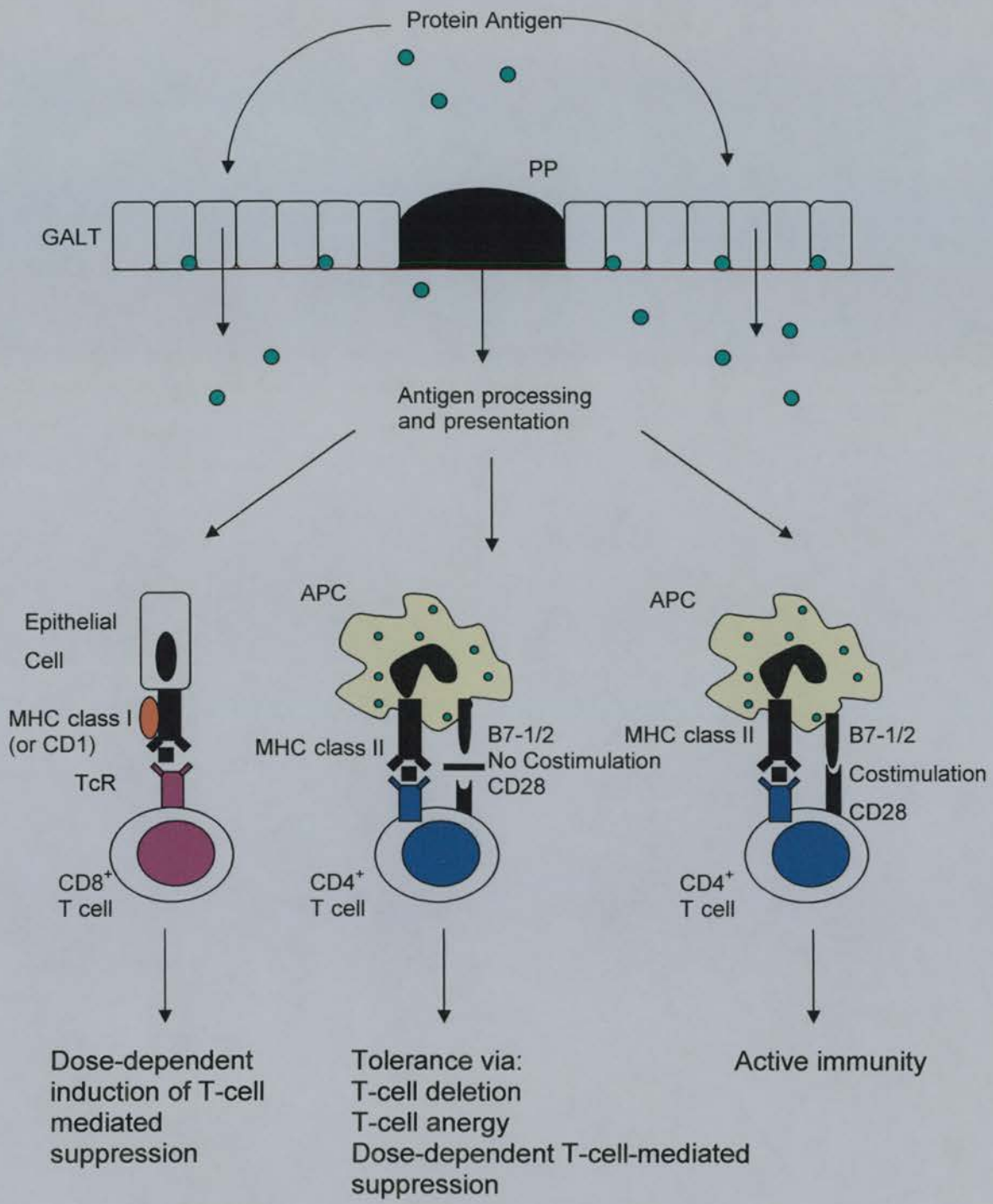


Fig. 1.4. Immunoregulation after oral antigen administration.

(Adapted from Strobel and Mowat [438])

Chapter 2

Materials and Methods

2.1 Purification of Der p 1

The house dust mite allergen Der p 1 (*D. pteronyssinus*) was affinity purified from spent mite medium (a kind gift from Dr. WR Thomas, Western Australian Institute for Child Health, Perth, Australia).

2.1.1 Preparation of the anti-Der p 1 antibody-coupled column

The 4C1 mAb (Indoor Biotechnologies Ltd, UK) recognises a cross-reacting epitope on all three Group I allergens (*Der p I, Der f I and Der m I*). Briefly, 100 mg 4C1 mAb was coupled to 7 g cyanogen bromide activated Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden #17-0430-01) according to manufacturer's instructions. The 4C1-sepharose was poured into a 20 ml column and equilibrated with PBS (pH 7.4).

2.1.2 Preparation of Mite Extract

Typically, the mite extract was prepared by extracting 100-150 g of the spent mite medium in 1 litre of PBS by stirring overnight at 4°C. The extract was centrifuged for 25 minutes at 3000 rpm and the pellet discarded. The supernatant was then passed through successive (5μm, 3μm, 0.8μm and 0.45μm) nitrocellulose filters (#7195 004, #7193 004, #7188 004, #7404 004, Whatman International, Maidstone, UK) under suction.

2.1.3 Extraction of Der p 1

The filtered supernatant was applied to the 4C1 column at a flow rate of

~20 ml/hour. The column was then washed with 400 ml PBS containing 0.5M NaCl. The bound Der p 1 was eluted with dH_2O (pH 11 with ammonium hydroxide, A-6899). Fractions (2.2 ml) were collected into tubes containing 0.8 ml 0.2M Na_2HPO_4, pH 7 at a flow rate of 20 ml/hour. The column was re-equilibrated with 400 ml of PBS containing 0.01% NaN_3 (S-2002) to prevent bacterial growth.

The absorbance of each fraction was measured at 280 nm to assess the concentration of Der p 1. Fractions containing Der p 1 were pooled and dialysed extensively against PBS (Oxoid). After dialysis the allergen was concentrated using Aquacide (Calbiochem, La Jolla, CA, USA). Aliquots of Der p 1 were stored at -20°C until use.

2.1.4 Assessment of Purity

The purity was assessed by SDS-PAGE on a 13% acrylamide gel. A resolving gel (see materials and buffers) was poured into the apparatus. Butanol (500 µl) was layered onto the gel to ensure that the gel remained level. After the gel had set, the butanol was washed off with dH_2O and a stacking gel (see materials and buffers) was then applied to the top of the apparatus. A plastic comb was pushed into the gel and left to set.

Der p 1 (5 or 10 µg) was diluted in 10 µl PBS and 10 µl of reducing loading buffer (see materials and buffers) added. Molecular weight (MW) markers (Gibco BRL, Paisley, U.K.) were also prepared by diluting 2 µl of MW markers in 10 µl PBS, and then adding this to 10 µl loading buffer. The samples and MW markers were boiled for 5 minutes.

The gel was placed in a gel tank filled with SDS-Tris Glycine electrophoresis buffer (see materials and buffers). The comb was removed and samples and MW markers added to the wells. A voltage of 100 volts was applied for 30 minutes followed by 200 volts until the dye front was close to the bottom.

The gel was stained with Coomassie brilliant blue (B-5133) buffer and destain see materials and buffers).

2.2 Immunisations

2.2.1 Mice

Female, 6-8 week-old, C57BL/6 and Balb/b mice were either obtained in house or from Harlan Olac Ltd. (Bicester, UK). They were housed in specific pathogen free conditions, and given food and water *ad lib*.

2.2.2 Generating a Th2-type response to Der p 1

2.2.2.1 Adjuvant Protocol

Imject® Alum (Pierce, Chester, UK) – an aqueous solution of aluminium hydroxide (45 mg/ml) and magnesium hydroxide (40 mg/ml) was used to generate a Th2-type response. Briefly, 100 μl of alum was added dropwise to 10 μg Der p 1/100 μl saline and then shaken for 30 minutes to enable effective adsorption of the immunogen. Mice were immunised s.c. on day 0 and then i.p. on days 14 and 28. The dose volume in each case was 200 μl containing 2 μg Der p 1.

2.2.2.2 Non-Adjuvant Protocol

Mice were immunised s.c. on day 0 with 10 μg Der p 1 in saline and then i.p. on days 2, 4, 6, 8, 10, 12 and 14 with the same amount. On days 34, 37 and 40, mice were given 25 μg Der p 1 in 50 μl saline by intra-tracheal instillation to induce lung eosinophil infiltration. Mice were anaesthetised with 250-300 μl avertin (i.p.) and the upper jaw rested over a wire. A small weight was placed on the lower jaw to open the airways and a bevelled 25G needle was placed into the trachea where upon the chosen solution was administered. Mice were then sacrificed on day 41.

A more detailed comparison of the two methods of immunisation for Th2 induction is shown in the next chapter.

General comment about the protocols adopted within this thesis

Throughout this thesis there was not always consistency in the protocols that were adopted. Although every effort was taken to try and make the experiments comparable, this was not always possible due to time constraints and limited supplies of high quality, low endotoxin Der p 1. The protocols adopted are described in detail throughout the thesis with reasons given for any deviation from standardised protocols.

2.2.3 Collection of serum

Blood was collected into eppendorfs where it was left at room temperature for 30 minutes and then at 4°C for at least an hour to allow clot formation. Blood was removed from the clot and centrifuged for 5 minutes at 5000 rpm. Sera was then removed from the red blood cell pellet and frozen at -70°C until use.

2.3 Measurement of Lung Eosinophilia

2.3.1 BAL Fluid

Mice were anaesthetised with 0.5 ml avertin i.p. The peritoneum was carefully opened to avoid damage to the diaphragm and the mice killed by cutting the para-aortic artery. The diaphragm was carefully cut and then the thorax opened. The lungs and trachea were carefully removed from the thoracic cavity to avoid puncture. A 3 cm length of plastic tubing was placed in the trachea and held by forceps whilst 0.5 ml of ice cold saline injected into the lungs. These were squeezed gently a few times and the BAL fluid removed and placed on ice. The BAL was centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and frozen at -20°C for later cytokine analysis by ELISA. The cell pellet was washed with PBS and then resuspended in 0.5 ml PBS.

2.3.2 Cytospins

The resuspended cell pellet (100 µl) was added to a poly-L-lysine (PLL)-coated glass slide and was spun at 300 rpm for 3 minutes. The slide was air-dried o/n.

2.3.3 Staining of Cytospins

To evaluate the levels of eosinophils in BAL samples, cytospin slides were fixed for 2 minutes in methanol. Slides were removed and placed in Diff-Quick I solution (eosin G in phosphate buffer – pH 6.6) for 3 minutes after which they were placed in Diff-Quick II solution (thiazine dye in phosphate buffer pH 6.6) for 2 minutes as a counterstain. The slides were then washed in approximately 5 changes of H_20 until the water appeared clear. Slides were left to air-dry before viewing under the microscope. Diff-Quick solutions were obtained from Dade Behring, CH-3186, Dűdingen.

2.3.4 Lung Tissue

Once the BAL fluid had been removed from the lungs they were placed in ice cold PBS. They were blotted dry on tissue paper and weighed. The tissue was then chopped into small pieces and made up to a 5% suspension in Hank's Balanced Salt Solution (HBSS) (without phenol red, calcium or magnesium) containing 10 mM HEPES (pH 7.4) and homogenised using a Polytron homogeniser. The homogenate was centrifuged for 10 minutes at 3000 rpm. The supernatant was discarded and the cell pellet was lysed by hypertonic shock using ice-cold buffers (0.2% NaCl for 10 seconds and then 1.6% NaCl/Dextrose). The homogenate was centrifuged again (10 minutes at 3000 rpm), the supernatant discarded and the pellet resuspended to a 5% suspension in HBSS/HTAB (HBSS containing 0.5% HTAB, pH 7.4). This was homogenised again and freeze thawed three times in liquid nitrogen and warm water. Samples were stored at -20°C until assayed.

2.3.5 Eosinophil Peroxidase Assay

The homogenates were centrifuged for 5minutes at 3000 rpm. Each supernatant (75 µl) was placed in a 96 well flat-bottomed plate and diluted to ½ and ¼ in HBSS/HTAB. A standard curve using eosinophils isolated from the lungs of IL-5 transgenic mice [559] was included. Substrate (150 µl) was added to each sample

and left at room temp. in the dark for 30 minutes. Plates were read at 490 nm in a Biorad ELISA reader.

2.4 Cell Culture

2.4.1 Cell Culture Medium

Cells were cultured with complete RPMI cell culture media. (see "Materials and Buffers" for composition).

2.4.2 Incubation

Cells were kept in an incubator at 37°C, 95% humidity and in an atmosphere of 5% CO_2 in air.

2.4.3 Preparation of cell suspensions

2.4.3.1 Spleen

Spleens were excised from mice and collected into ice-cold HBSS (containing P/S). Tissues were macerated between 2 frosted glass slides until a homogenous cell suspension was obtained. The suspension was placed in 15 ml Falcon tubes and left to settle for 5 minutes, after which the supernatant was transferred to a clean tube and centrifuged for 7 minutes at 1400 rpm. The supernatant was discarded and the pellet resuspended with 1 ml ice cold Tris-NH_4Cl red blood cell lysis buffer and left on ice for 5 minutes. Complete media (4 ml) was added and suspension centrifuged again for 7 minutes at 1400 rpm. The supernatant was discarded and the pellet resuspended in 5 ml complete media and left on ice until use. Cell suspensions were counted using a haemocytometer chamber with trypan blue exclusion.

2.4.3.2 Lymph Nodes and Peyer's Patches

Lymph nodes and Peyer's Patches were pressed through a 70 μm sterile gauze by a plunger of a 1 ml plastic syringe into 5 ml HBSS (containing P/S). This was transferred to a 5 ml Falcon tube and centrifuged for 7 minutes at 1400 rpm. The

supernatant was discarded and the pellet was resuspended in 1 ml complete media. Cells were counted as described above.

2.4.4 Restimulation of Cells

For cell proliferation and cytokine determination, cells were cultured in triplicate at a density of 5 x 10^5 in 200 µl per well in 96-well flat-bottomed plates (for spleen) and at 2 x 10^5 in 200 µl per well in 96-well round bottomed plates (for lymph nodes and Peyer's Patches). Antigens were added to the cultures at the concentrations indicated. As a positive control, cells were either stimulated with 1 µg/ml Concanavalin A or anti-CD3/CD28. For anti-CD3/CD28 stimulation, 96 well flat-bottomed plates were coated with anti-CD3 in sterile PBS (50 µl/well at 5 µg/ml) and left at 37°C for 1 hour. The plates were washed thoroughly with PBS (200 µl/well x 3). Cells were then cultured with 2.5 µg/ml anti-CD28.

2.5 Measurement of Proliferation

Earlier experiments involve the addition of 0.5 µCi/well of [3H]-thymidine (ICN, Basingstoke, Hampshire, UK) at 66 hours. Plates were then harvested or frozen six hours later. Later experiments added [3H]-thymidine at 72 hours and an incubation of 18 hours before freezing or harvesting. The figure legends make clear which thymidine protocol was used. The cells were harvested onto fibreglass filters (Wallac, Crownhill, Buckinghamshire, UK) using a 96 well Tomtec plate harvester (Wallac). The filter mat was sealed into a plastic bag (Wallac) with 10 ml of 'Betaplate™ Scint' liquid scintillation fluid (Fisons, Loughborough, Leicestershire), placed in a holding cassette and counted on a 1205 Betaplate™ scintillation counter (Wallac) allowing 20 seconds of counting for each sample.

2.6 ELISA (Enzyme Linked Immunosorbant Assay)

2.6.1 Cytokine Detection From Cell Culture Supernatants

Cytokine secretion was analysed by detecting soluble protein aliquots of supernatants collected from cultures at 48 hours (for IFN-γ,) and 72 hours (for IL-5, IL-13 and IFN-γ). Figure legends make clear at which time supernatants were removed.

Briefly, anti-cytokine capture antibodies were diluted to the appropriate concentration (see table on page 92) in ELISA binding buffer and added (50 µl/well) to 96 well flat-bottomed plates with enhanced protein binding capacity (Corning, NY, USA). The plates were sealed and incubated overnight at 4°C.

The plates were washed 3 times with ELISA wash buffer and blotted dry on tissue. Blocking buffer A was added to each well (200 µl) and left at room temperature for 1 hour.

Plates were washed as before, the samples and standards (diluted in blocking buffer A) added (50 µl/well) and incubated overnight at 4°C.

The plates were washed again (three short washes and two longer washes) and 50 µl of biotinylated detection antibody (diluted in blocking buffer A) at the appropriate concentration was added to each well and then incubated for two hours at room temperature.

Plates were washed again (three short and two long washes) before the addition of Streptavidin-alkaline phosphatase conjugate (Amersham Life Sciences, Amersham, UK); this was diluted 1:2000 and 50 µl was added to each well. The plates were incubated at room temperature for 30 minutes.

Plates were washed again (three short and two long washes) and 100 µl substrate solution was added to the wells. The plates were left to develop in the dark and were read at different time intervals (between 20 – 60 minutes depending on how quickly the reaction developed) on a Microplate Reader 450 (Biorad Laboratories, Hemel Hempstead, UK).

2.6.2 Cytokine/Chemokine ELISA reagents:

Cytokine/ Chemokine Detected	Capture Antibody	Conc. Capture Antibody	Biotinylated Detection Antibody	Conc. Biotinylated Antibody	ELISA Standard
IFN-γ	18181D Pharmingen	1 μg/ml	18112D Pharmingen	1 μg/ml	19301U Pharmingen
IL-5	18051D Pharmingen	1 μg/ml	18062D Pharmingen	1 μg/ml	Gift from S. Hirschberg
IL-13	MAB413 R&D	2 μg/ml	BAF413 R&D	0.2 μg/ml	413ML005 R&D
IL-4	18031A Pharmingen	1 μg/ml	18042D Pharmingen	1 μg/ml	19231V Pharmingen
IL-9	18461D Pharmingen	2 μg/ml	18452D Pharmingen	1 μg/ml	554584 Pharmingen
Eotaxin	AF420NA R&D	1 μg/ml	BAF420 R&D	50 ng/ml	420-ME R&D

Pharmingen, San Diego, CA, USA

R&D Systems, Abingdon, Oxford

Later experiments used the Duoset ELISA Development System because of increased sensitivity. The assay was developed according to the manufacturer's instructions (R & D Systems, Abingdon, Oxon).

Mouse IL-4	#DY404
Mouse IL-5	#DY405
Mouse IL-10	#DY417
Mouse IL-13	#DY413
Mouse IFN-γ	#DY485

2.6.3Antibody Detection From Mouse Sera

2.6.3.1 Der p 1-Specific IgG1 and IgG2a

The ELISA methodology outlined above was undertaken for detection of Der p 1-specific IgG1 and IgG2a but plates were initially coated with Der p 1 (in place of the capture antibody) at 5 μg/ml. Sera was added to the plates at concentrations of 1:10, 1:100 and 1:1000, in duplicate (50 μl/well). A standard curve was included using either purified mouse IgG1 (Pharmingen, 03001D) or IgG2a (Pharmingen, 03021D). Standards were diluted in blocking buffer from 500 ng/ml to 0.5 ng/ml. Biotinylated IgG1 (MCA336B, Serotec Ltd, Oxford, UK) or biotinylated IgG2a (MCA421B, Serotec) were used at 1 μg/ml.

2.6.3.2 Der p 1-Specific IgE

ELISA plates were coated with purified anti-mouse IgE (Pharmingen #02111D) at 2 μg/ml in binding buffer (50 μl/well) and were left o/n at 4°C. The plates were washed 3 times with ELISA wash buffer and blotted dry on tissue. Blocking buffer (3% BSA in PBS) was added to each well (200 μl) and left at room temperature for 3 hours. Plates were washed again as before, and sera was added (1:10, 1:20, 1:40) in 1% BSA/PBS (50 μl/well), a positive control was included (from mice previously immunised with Der p 1/alum and known to have high IgE levels) as well as a commercial standard (Pharmingen #03131D) diluted from 500 ng/ml to 1 ng/ml. The plates were incubated overnight at 4°C. The plates were washed four times. Der p 1 was added to all wells with the exception of those containing commercial standard at 10 μg/ml (50 μl/well). Biotinylated anti-mouse IgE (Pharmingen #02112D) was added to wells containing commercial standard at 1 μg/ml (50 μl/well). The plates were incubated at 37°C for two hours. After four washes, biotinylated anti-Der p 1 (4C1 Indoor Biotechnologies Ltd, UK) was diluted 1:200 in1% BSA in PBS and added (50 μl/well) to all wells except the commercial standard. PBS/1%BSA was added to wells containing commercial standard to stop them from drying out. Plates were incubated at 37°C for 1 hour. The plates were washed five times before the addition of 100 μl/well of Avidin-Horseradish Peroxidase (Pharmingen #554058)

diluted 1:1500. Plates were incubated for 30 minutes at room temperature. The plates were washed for a total of six times including two soaking steps of at least one minute. Substrate solution (R & D systems, #DY999) (100µl/well) was added to the plates. This involved mixing Substrate A (H_2O_2) and Substrate B (tetramethylbenzidine) (1:1 ratio) before addition to plates which were then left in the dark for up to 30 minutes for the reaction to proceed. The reaction was stopped with 50 µl/well of 0.5M H_2SO_4. The optical density of each well was determined using a microplate reader set to 450 nm.

2.6.3.3 Total IgE

The Der p 1-specific determination of IgE was only developed recently and so earlier experiments measured total IgE determination.

The 'OptEIA™ Mouse IgE Set (2655K1, Pharmingen) was used to determine total IgE. The assay was carried out according to the manufacturer's instructions but briefly: plates were coated with 2 µg/ml of capture antibody (50 µl/well). The plates were sealed and incubated overnight at 4°C. The plates were washed three times with washing buffer. The plates were blocked with 200 µl of blocking buffer and left at room temperature for 1 hour. The plates were washed as before. The standard was added to the plates at 50 µl well from 200 ng/ml to 3.1 ng/ml. The sera samples were diluted 1:10, 1:20 and 1:100 and added to the plates in duplicate (50 µl/well). The plates were incubated for 2 hours at room temperature. The plates were washed five times in total (three quick washes and two longer washes). The detection antibody (at 1 µg/ml and 50 µl/well) was added to the plates along with the streptavidin-horseradish peroxidase conjugate. The plates were incubated at room temperature for a further hour. The plates were washed five times in total (three quick washes and two longer washes). Substrate solution (Tetramethylbenzidine and hydrogen peroxide) was added (100 µl/well). Plates were read at 450 nm after 30 minutes.

2.7 Materials and Buffers

All chemicals were purchased from Sigma (Dorset, U.K.) unless otherwise stated.

Der p 1 Peptides

All of the Der p1 peptides were manufactured by Ian Moss, Advanced Biotechnology Centre, Charing Cross Hospital, London.

The following peptides were made:

Der p 1;p110-131:	**RFGISNYCQIYPPNANKIREAL**
Der p 1;p111-139:	**FGISNYCQIYPPNANKIREALAQPQRYCR**
Der p 1;p113-127:	**ISNYCQIYPPNANKI**
Der p 1;p111-119:	**FGISNYCQI**

In H-2^b mice, the immunodominant epitope of Der p 1 is Der p 1;p110-131. This sequence contains both the MHC class I (p111-119) and MHC class II-binding (p113-127) regions.

MEA Particles

These particles were provided by ALK (Hørsholm, Denmark).

Der p 1 or Der p 1;p111-139 were encapsulated within the particles which consisted of a polymer – poly-lactide-co-glycolide (PLG). The ratio of lactide to glycolide was 60:40. Particles were visualised by electron microscopy and were found to be < 10 μm.

Avertin (Tribromomethanol anaesthetic)

For 200 ml:

2.5 g 2,2,2-tribromomethanol (T4840-2)

5 ml 2 methyl-2-butanol (tertiary amyl alcohol) (A1685)

200 ml H_2O

Preparation:

1. The tribromoethanol was mixed with the alcohol on a heated stirrer until all the crystals had dissolved.
2. Distilled H_20 was added until completely dispersed.
3. The avertin was aliquoted and stored in the dark at 4°C for up to a year.

Use:

The avertin was warmed to 37°C for half an hour before use.

Complete Mouse RPMI culture media

Complete culture medium (500 ml), made from RPMI-1640 (R-0883, Gibco, Paisley, UK) supplemented with 2 mM L-glutamine (Gibco, Paisley, U.K., 25033-010), 100 μg/ml penicillin/streptomycin (Gibco, Paisley, U.K. 15070-071), 5 x 10^{-5} M β-mercaptoethanol (M-7522) and 5% foetal calf serum (#4-101-500, Labtech International, www.labtech-international.com). Kept at 4°C.

Coomassie Destain

10% glacial acetic acid (A-6283)

40% methanol (M-1770).

Coomassie Stain (1 L)

10% glacial acetic acid (A-6283)

40% methanol (M-1770)

1 g Coomassie brilliant blue (B-5133).

ELISA Binding Buffer

Carbonate-Bicarbonate buffer (C-3041). 1 capsule dissolved in 100 ml of dH_2O yields a 50 mM Carbonate-Bicarbonate buffer, pH 9.6.

ELISA Blocking Buffer

1% bovine serum albumin (A-9418)

5% sucrose (S-7903)

0.5% NaN_3 (S-2002)

in Dulbecco's PBS (D-8537)

ELISA Wash Buffer

PBS (Oxoid, Ltd, Basingstoke, UK) containing 0.05% Tween 20 (P-1379).

ELISA Substrate Solution C (for non-Duoset ELISA kits)

ELISA ethanolamine buffer (Don Whitley Scientific, Shipton, U.K. E-016) containing 1 mg/ml pNPP (N-2770). Made fresh.

PLL-Coated Glass Slides

Poly-L-lysine solution (P-8920) was dissolved 1:10 in dH_2O. Slides were incubated in the solution for five minutes at room temperature before drying o/n.

Reducing Loading Buffer

50 mM Tris-HCl (pH6.8) (T-3253)

2% SDS (L-4509)

0.1% bromophenol blue (B-5525)

5% β-mercaptoethanol (M-7522)

10% glycerol (BDH #110184K)

Red Blood Cell Lysis Buffer (Gey's Lysis Buffer)

8.29 g NH_4Cl(A-5666)

1 g $KHCO_3$ (P-9144)

200 µl 0.5M EDTA

in 1 litre dH2O.

Sterile filter and keep at 4°C.

Resolving SDS-PAGE Gel

1.7 ml 40% Bis-acrylamide (A-3699)

1.9 ml 1 M Trizma Base pH 8.8 (T-1503)

1.4 ml ddH_2O 1 ml

50 µl 10% SDS (L-4509)

Polymerisation of 5mls gel mixture catalysed by addition of 5 µl TEMED (T-9281) and 50 µl 10% ammonium persulphate (A-3678).

Stacking Gel

402 µl 40% Bis-acrylamide (A-3699)

375 µl 1M Trizma Base pH 8.8 (T-1503).

2.2 ml ddH_2O

30 µl 10% SDS (L-4509)

Polymerisation of gel mixture was catalysed by the addition of 3 µl TEMED (T-9281) and 30 µl 10% ammonium persulphate (A-3678).

SDS-Tris Glycine Electrophoresis Buffer

30 ml 1M Trizma Base (T-1503)

20 ml 1M Tris-HCl (T-3253)

28.8 g Glycine (G-7403)

20 ml 10% SDS (L-4509)

Made up to 2000 ml with ddH_2O

Chapter 3

Induction of a Th2 response in mice without the use of adjuvant.

3.1 Introduction

The aim of this study was to use an animal model for investigating potential therapeutical approaches for modulating established Th2 responses. Alum is the adjuvant of choice in therapy currently in use but we wanted to develop an alum-free model. The reasons for this were threefold. Firstly we wished to adopt a more physiologically relevant approach for inducing Th2 responses, secondly, we were concerned that subtle differences in immune modulation might be difficult to detect because alum induces such strong responses, finally, adjuvants by themselves induce a nonspecific T cell activation as well as inflammatory responses and affect several cellular functions [439, 440]. Hence the use of adjuvants may not be ideal for the study of allergic disorders.

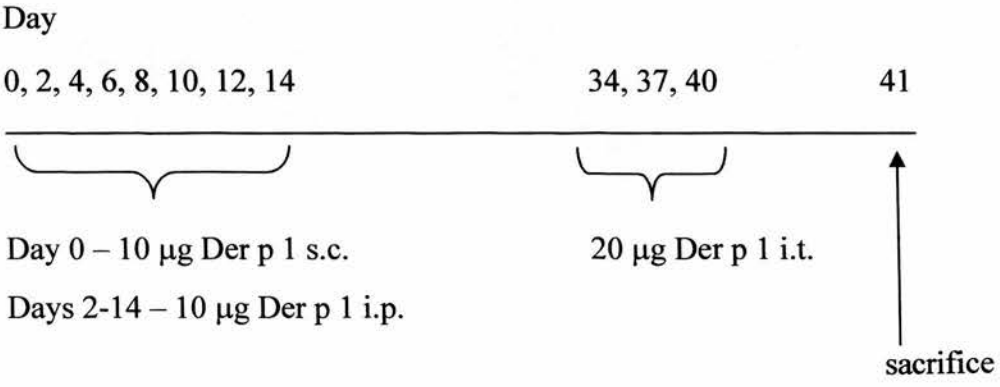
Protocols for the induction of systemic IgE responses to various allergens with accompanying airway hyperresponsiveness have previously been devised by other groups. These methods of sensitisation usually involve systemic immunisation followed by local challenge of the airways using nebulised allergen. This protocol was pioneered by Renz et al and subsequent studies have usually utilised part of this nebulisation procedure [441]. For example, painting the skin of Balb/C mice with an ovalbumin solution followed by one period of nebulisation, induced systemic IgE, IgG1 antibody responses, immediate cutaneous hypersensitivity and increased airway responsiveness [442]. Earlier work by Holt et al, showed that priming mice with ovalbumin intraperitoneally on a number of occasions without the use of adjuvant, induced specific IgE responses [443].

In this study, naïve female H-2^b (C57BL/6) mice were immunised using the protocol described on page 101 which was adapted from a study by Gavett and

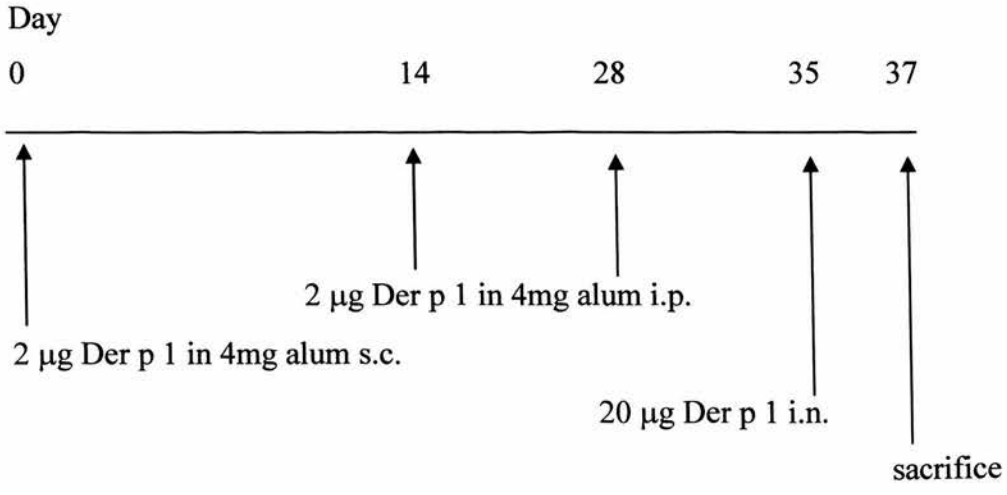
colleagues [584]. Briefly, mice were immunised systemically on repeated occasions with Der p 1 in saline and later rechallenged with Der p 1/saline intratracheally. Results were compared with mice that were immunised with Der p 1 in alum adjuvant using a conventional protocol (see protocol for specific details). The mice were sacrificed 24 hours after the last intratracheal challenge (for adjuvant-free) or 48 hours after intranasal challenge (Der p 1/alum). The draining lymph nodes (tracheal and cervical respectively), lungs and sera were removed from all animals. In addition, BAL fluid was removed *in-situ* from the adjuvant-free mice for the preparation of cytospins.

3.2 Protocols

Adjuvant-free Protocol



Der p 1/Alum Protocol



3.3 Results

Lymphocytes (2 x 10^5/well) were cultured in complete media either alone or supplemented with 1, 5 or 20 μg/ml Der p 1. Due to insufficient cell numbers for individual mice, lymph nodes were pooled within groups. Figure 3.1 shows lymphocyte proliferation from the adjuvant-free and Der p 1/alum mice. There is a dose-dependent increase in antigen-specific proliferation in the adjuvant-free mice compared to the Der p 1/alum treated mice. At maximal proliferation the difference between these groups is a six-fold increase.

There was no detectable IFN-γ in any of the culture supernatants apart from those restimulated with α-CD3/α-CD28 (results not shown), however, marked amounts of IL-5 were detected in lymphocyte cultures from the adjuvant-free mice and the levels paralleled proliferation (figure 3.2). Surprisingly, there was no detectable IL-5 in the mice that were injected with Der p 1 in alum.

The presence of IL-13 in lymphocyte culture supernatants was also measured as a second indicator of a Th2 phenotype. Only the adjuvant-free mice exhibited detectable levels of this cytokine which closely paralleled IL-5 levels (figure 3.3). Like IL-5, the Der p1 in alum treated mice exhibited no detectable IL-13 which was unexpected and cannot easily be explained.

Figure 3.4 shows the levels of IL-5 in BAL fluid taken from the mice. There was no detectable level of either cytokine for the saline treated mice in both groups but marked amounts were detected in the Der p 1-treated mice with and without the use of adjuvant. A similar trend in IL-4 production was also observed (fig. 3.5).

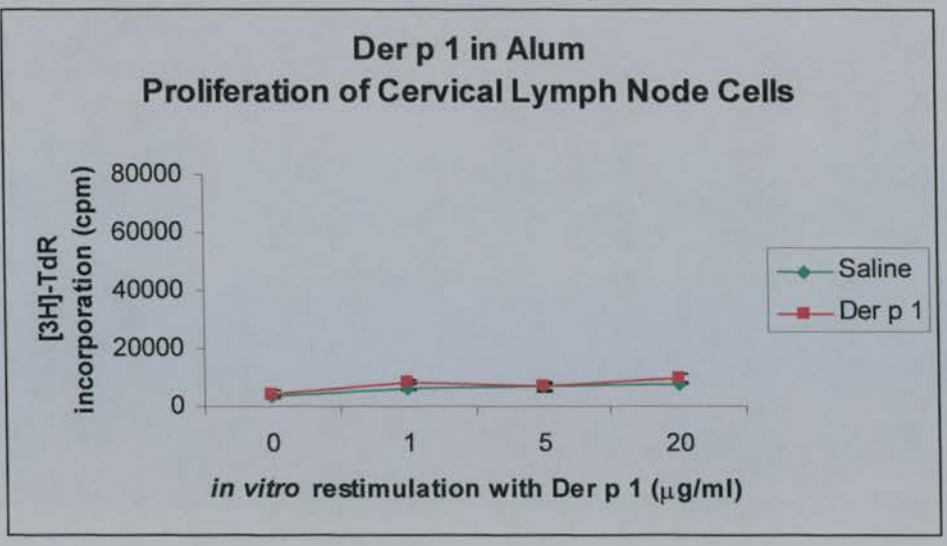
Extensive lung eosinophil infiltration was observed in both groups of Der p 1 treated mice. Despite some individual variations, there was little difference overall in eosinophilia between Der p 1/alum and the adjuvant-free mice (figure. 3.6).

Figure 3.7 shows photographs of cytospins of BAL fluid from the adjuvant-free mice. Eosinophils were presented as the dominant cell type (multi-lobed nuclei with red intracellular granules; Figure 3.7a). Figure 3.7b was a control cytospin using the BAL fluid from a mouse that had received saline alone intratracheally instead of

Der p 1/saline. Very few eosinophils were found to be present with the population consisting predominantly of macrophages (large mono-nuclear cells). No cytospins were prepared from the alum treated mice.

The presence of different isotypes of Der p 1-specific antibodies present in the sera is shown in figure 3.8. The predominant isotype in both groups is IgG1. Overall, the Der p 1/alum mice have more IgG1 but there are still marked levels of this isotype present in the sera of the adjuvant-free mice. Only small levels of IgG2a were observed in the Der p 1/alum mice with hardly any detectable IgG2a in the adjuvant-free mice. We were unable to detect any Der p 1-specific IgE in both the Der p 1/alum treated mice or the adjuvant free mice.

This experiment was carried out once due to time constraints and limited availability of Der p 1. The results achieved for the Der p 1 mice without adjuvant were thought to be good enough to use the same (or slight variations of the adjuvant free protocol) in later experiments.



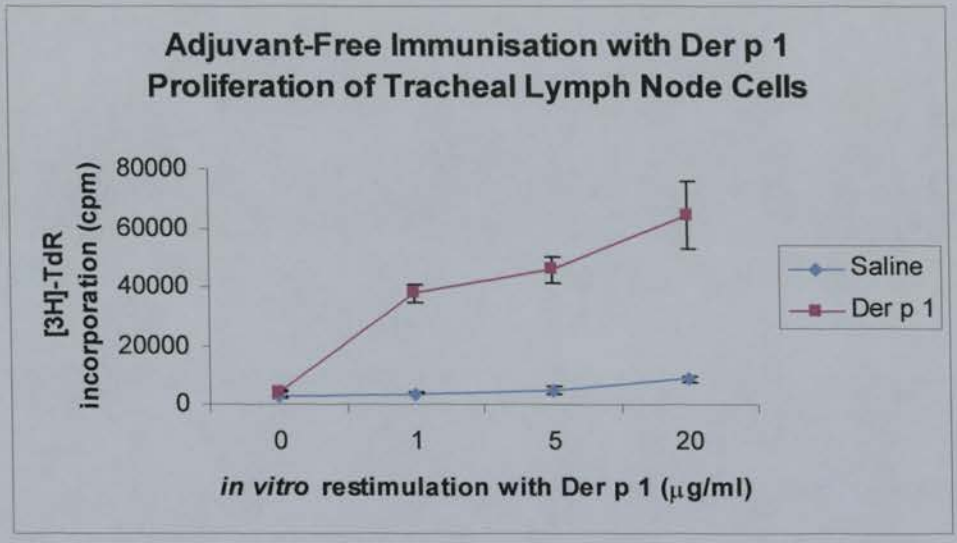
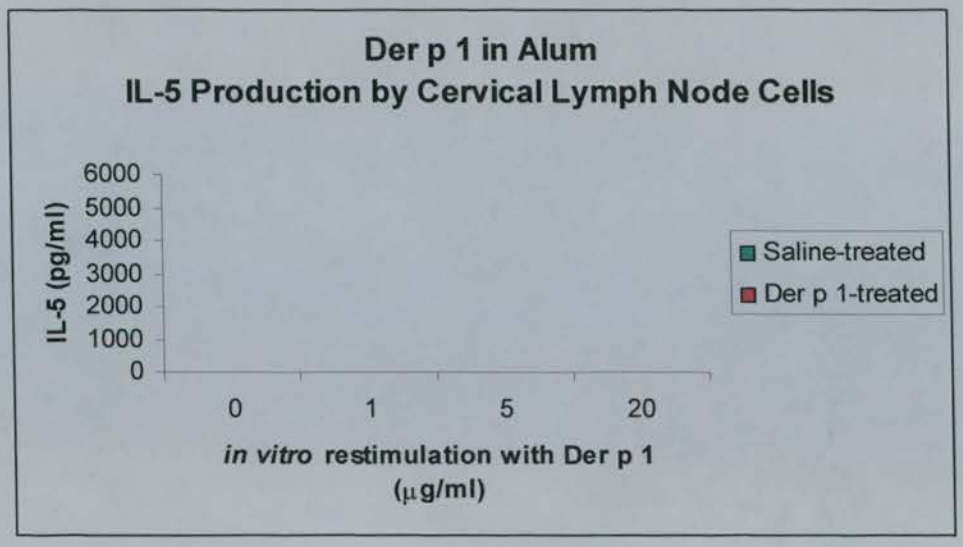


Figure 3.1. – Comparison of Der p 1 immunisations with and without the use of alum adjuvant.
Proliferation of draining lymph node cells.
Lymphocytes (2 x 10^5 per well) were restimulated *in vitro* with media alone or media supplemented with 1, 5 or 20 µg/ml Der p 1. Proliferation was measured at 72 hours after pulsing with thymidine at 66 hours. Lymph nodes were pooled within each group of mice: saline/alum (n = 5), Der p 1/alum (n =5), saline/adjuvant-free (n =3), Der p 1/adjuvant-free (n = 4).
Mean +/- SE is shown.



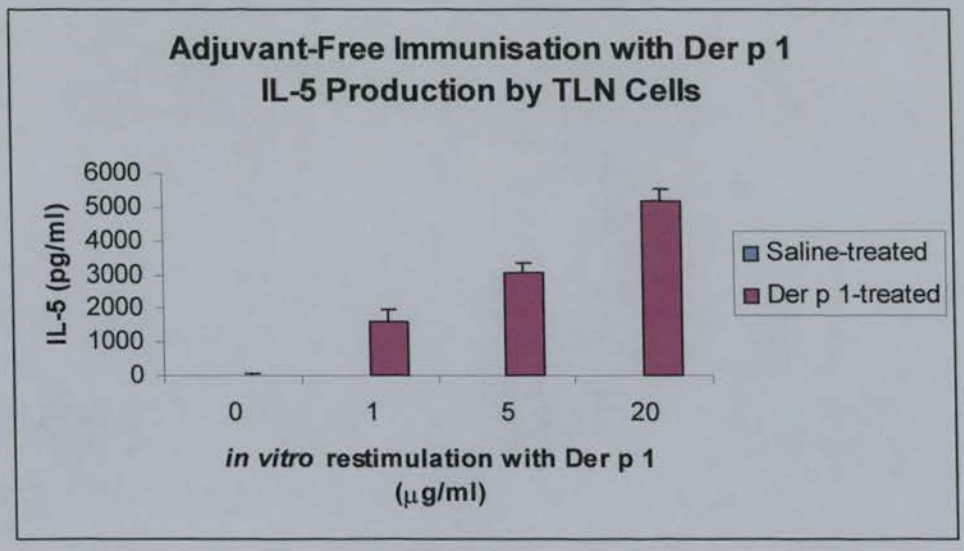
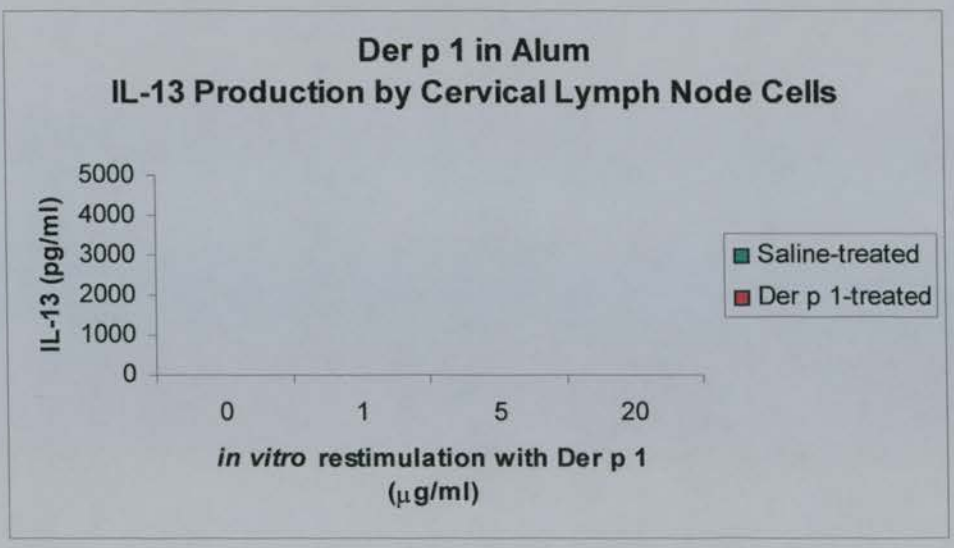


Figure. 3.2. – Comparison of Der p 1 immunisations with and without the use of alum adjuvant.
IL-5 levels in the draining lymph node cultures.
Lymphocytes (2 x 10^5 per well) were restimulated *in vitro* with media alone or media supplemented with 1, 5 or 20 μg/ml Der p 1. Supernatants were removed at 72 hours for the measurement of IL-5 by ELISA. Lymph nodes were pooled within each group of mice: saline/alum (n = 5), Der p 1/alum (n =5), saline/adjuvant-free (n =3), Der p 1/adjuvant-free (n = 4).
Mean +/- SE is shown.



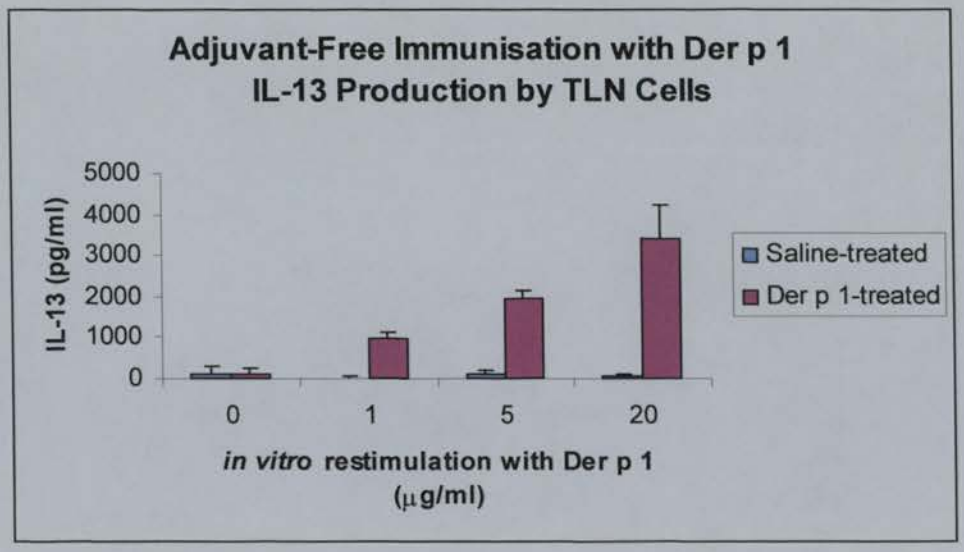
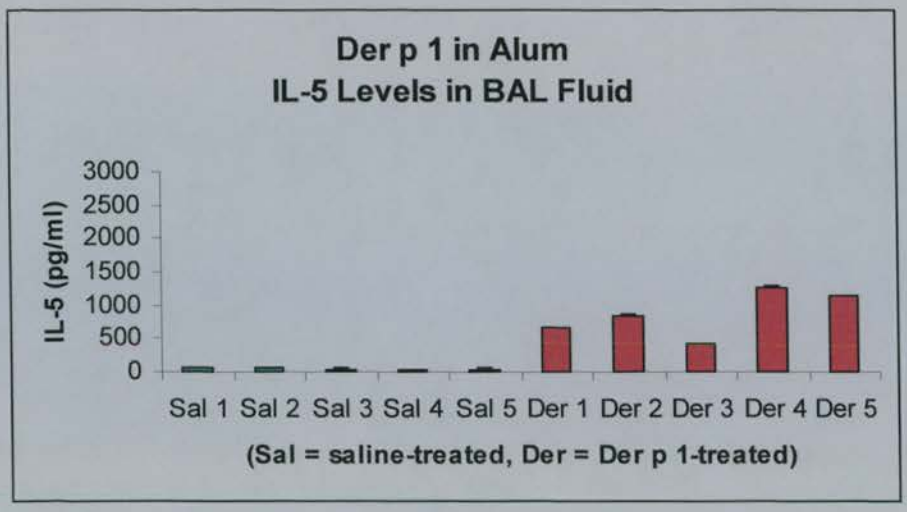


Figure 3.3. – Comparison of Der p 1 immunisations with and without the use of alum adjuvant.
IL-13 levels in the draining lymph node cultures.
Lymphocytes (2 x 10^5 per well) were restimulated *in vitro* with media alone or media supplemented with 1, 5 or 20 μg/ml Der p 1. Supernatants were removed at 72 hours for the measurement of IL-13 by ELISA. Lymph nodes were pooled within each group of mice: saline/alum (n = 5), Der p 1/alum (n =5), saline/adjuvant-free (n =3), Der p 1/adjuvant-free (n = 4).
Mean +/- SE is shown.



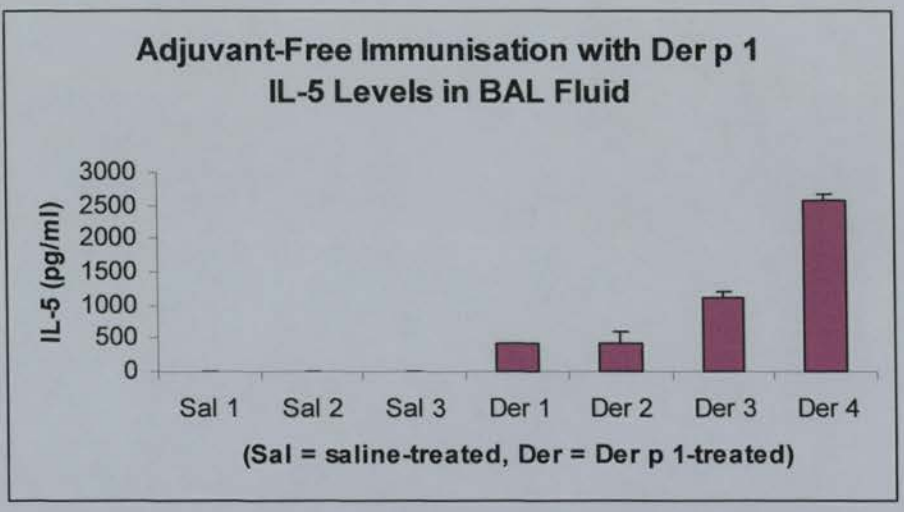
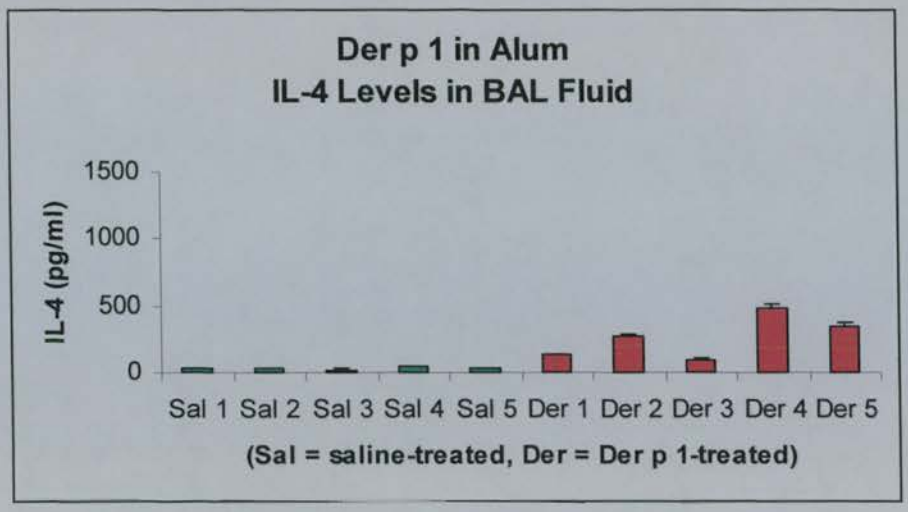


Figure. 3.4. – Comparison of Der p 1 immunisations with and without the use of alum adjuvant.
IL-5 Levels in BAL fluid.
Mouse lungs were lavaged in situ after death with PBS. The lavage was centrifuged to remove the cellular infiltrate (used for cytospins). The supernatant was frozen at -70° until analysis by ELISA.
The presence of IL-13 in BAL fluid was also examined but levels were beyond the threshold of detection.
Mean +/- SE is shown.



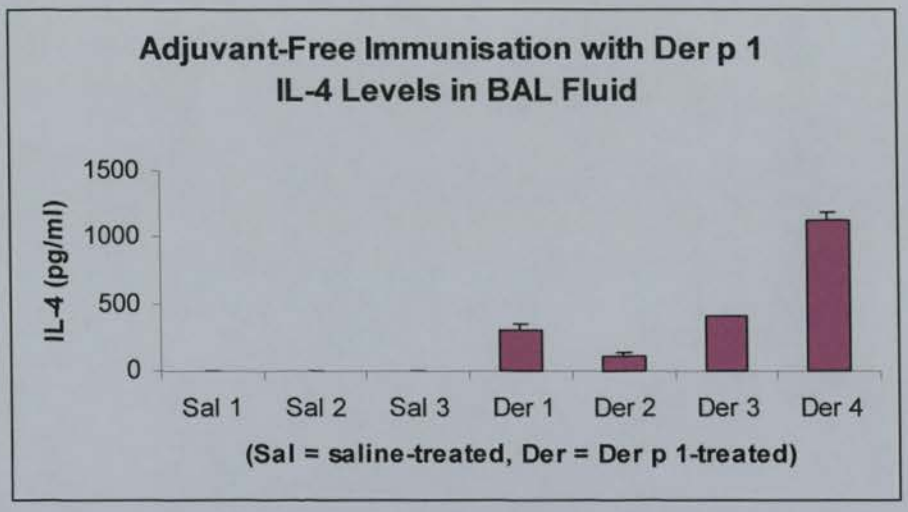
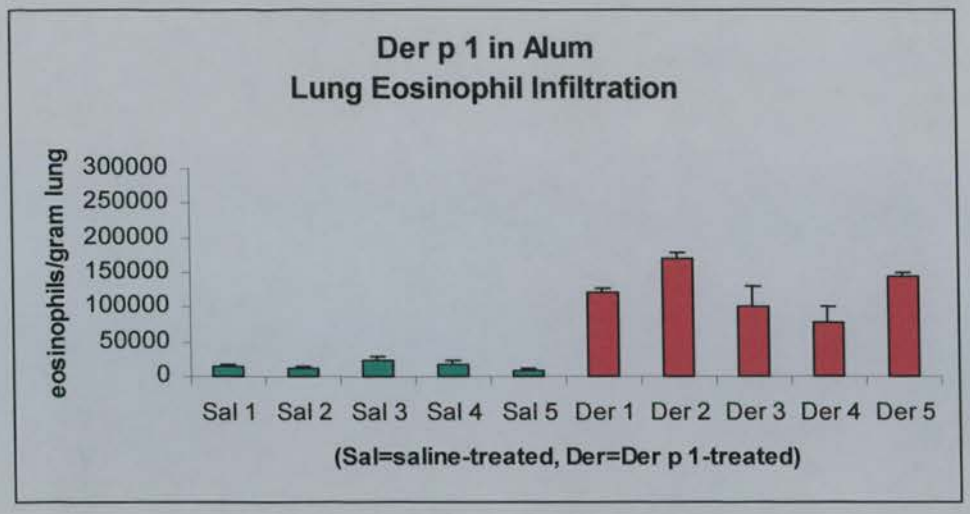
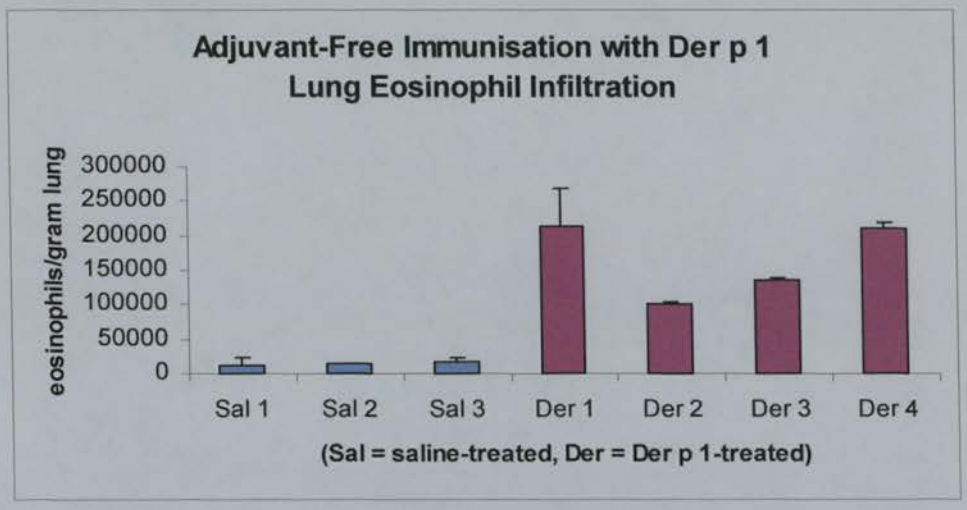


Figure. 3.5. – Comparison of Der p 1 immunisations with and without the use of alum adjuvant.
IL-4 Levels in BAL fluid.
Mouse lungs were lavaged in situ after death with PBS. The lavage was centrifuged to remove the cellular infiltrate (used for cytospins). The supernatant was frozen at -70° until analysis by ELISA.
The presence of IL-13 in BAL fluid was also examined but levels were beyond the threshold of detection.
Mean +/- SE is shown.





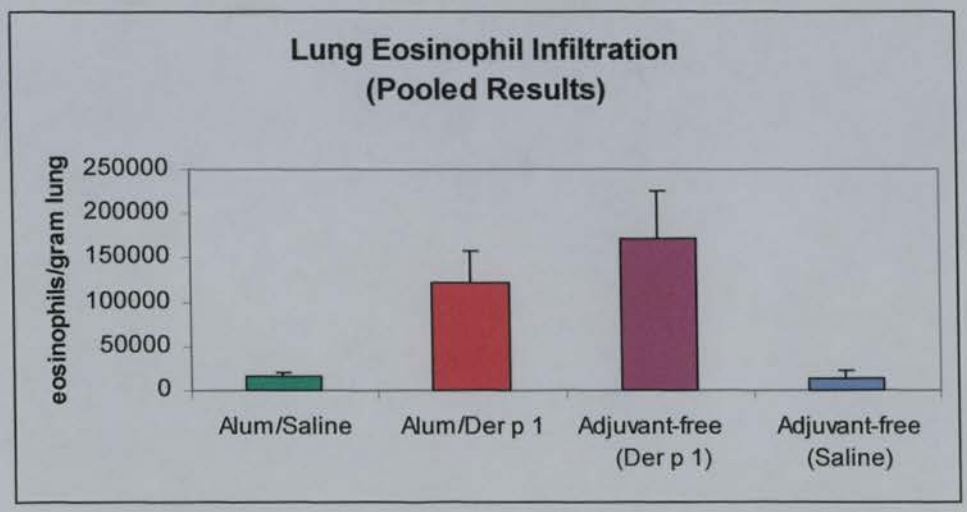


Figure 3.6. – Comparison of Der p 1 immunisations with and without the use of alum adjuvant.
Eosinophil infiltration into the lungs.
Eosinophils were detected by the eosinophil peroxidase assay.
Mean +/- SE is shown.

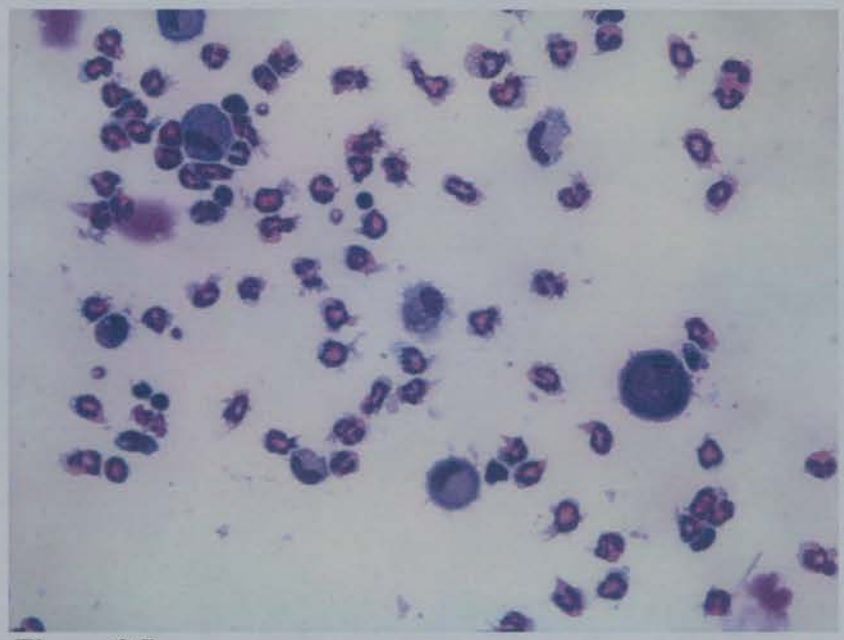


Figure 3.7a

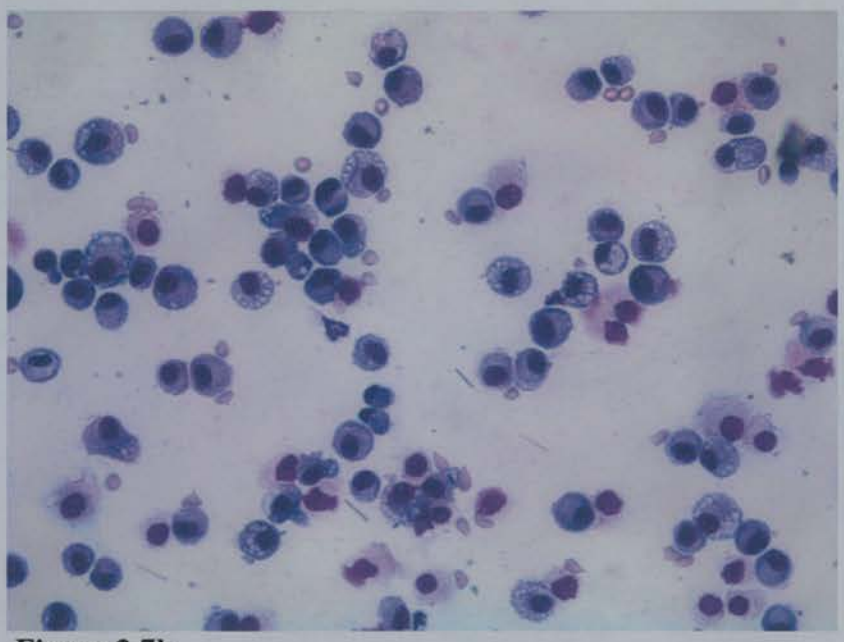


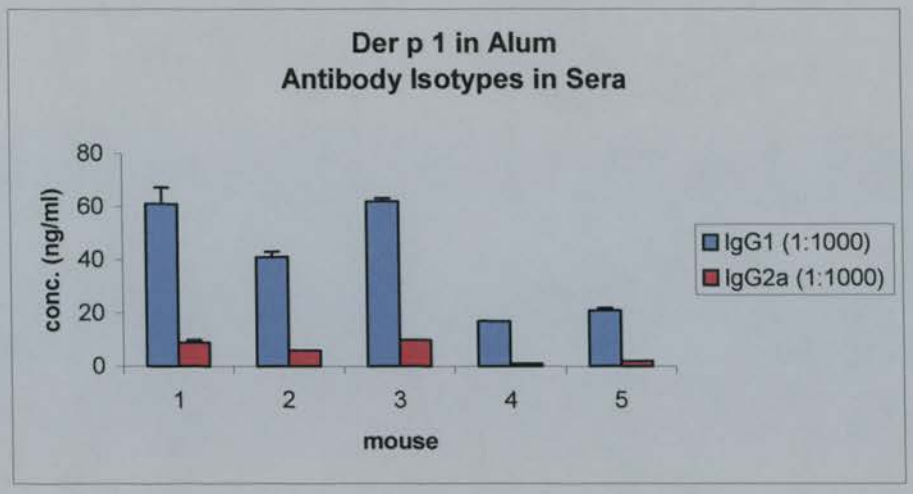
Figure 3.7b

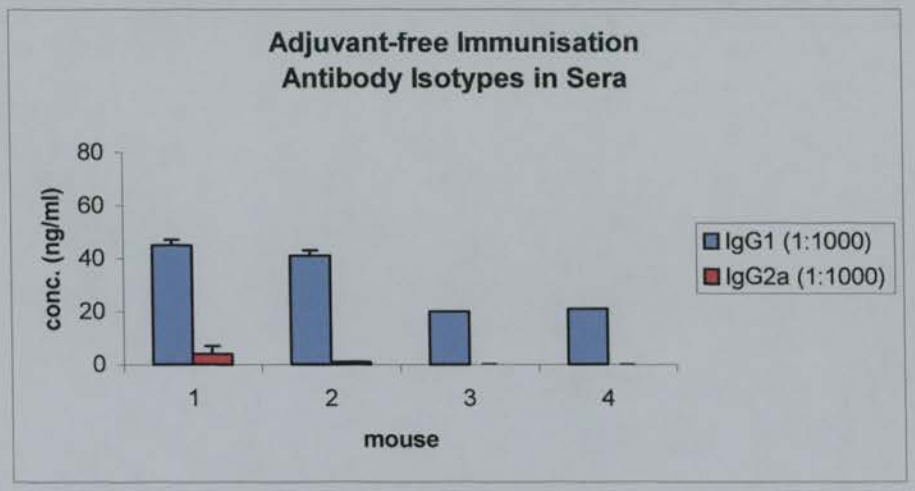
Figure. 3.7. – Eosinophil infiltration into the lungs of adjuvant-free mice – cytospins.

Cytospins were prepared from the BAL fluid and stained with Diff-Quick solutions (Eosin G (red) and Thiazine dye (blue)). Fig. 3.7a shows the cellular infiltrate from the lungs of mice immunised with Der p 1 intratracheally.

Fig. 3.7b shows the cellular infiltrate from the lungs of mice immunised with saline intratracheally.

Magnification was X100.





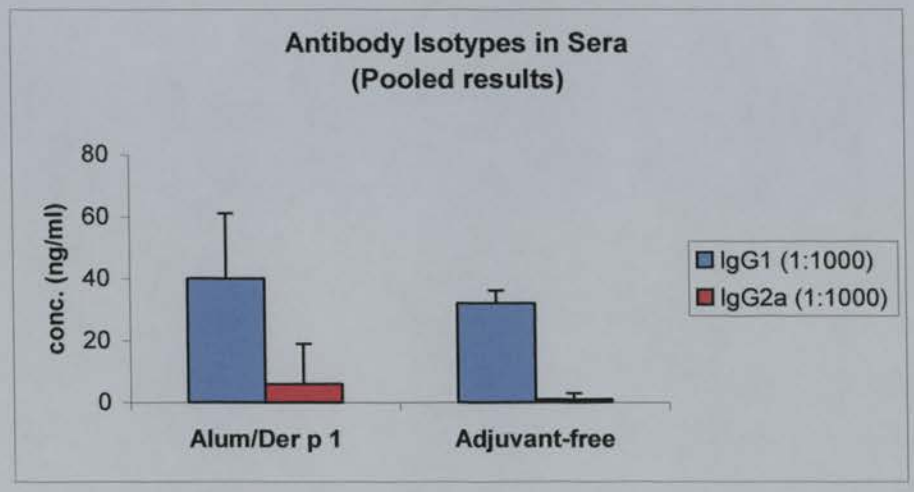


Figure 3.8. – Comparison of Der p 1 immunisations with and without the use of alum adjuvant.
Der p 1-specific IgG1 and IgG2a levels in sera.
Der p 1-specific IgG1 and IgG2a antibodies were detected by ELISA.
Mean +/- SE is shown.

3.4 Discussion

The aim of these experiments were to induce allergic Th2 inflammatory responses without the use of adjuvant. Traditionally, alum (aluminium hydroxide) has been used to enhance the immunogenicity of a protein and specifically promotes Th2-cytokine mediated immunity that is usually indicated by the presence of IgG1 and/or IgE in the sera of animals [444-446]. In another study, it was reported that eosinophilia was related to the dose of alum rather than the antigen adsorbed to it, and the authors proposed that alum had a direct effect on T cells, causing them to secrete chemotactic factors for eosinophils [447]. Alum enhances the immunogenicity by converting the soluble protein antigen into particulate material, which is more readily ingested by antigen-presenting cells such as macrophages. It also creates a depot effect by slowly releasing the protein or peptide over a period of time.

However, we were concerned that using alum to induce Th2 immunity to Der p 1 was unphysiological and unrepresentative of natural exposure to allergen which occurs in the absence of adjuvant. In addition, the strength of Th2 responses induced by antigen delivered in alum might make it difficult to detect subtle changes in phenotype mediated by potential therapeutic agents.

Adjuvant-free protocols are not a new concept. As discussed in the introduction, Renz et al. sensitised mice to OVA by nebulisation. Whilst this immunisation schedule was perfectly adequate, the amount of antigen required to sensitise the mice was quite large. This was acceptable in a study utilising ovalbumin (a fairly innocuous antigen) as the antigen of interest but our own antigen (Der p 1) was not as readily available and more importantly posed a safety hazard when nebulised, thus, we adopted a protocol where antigen wastage and hazard to health were minimal.

Thus, an immunisation protocol was established in house that could induce allergen-specific Th2 responses both systemically and locally in the airways, by the administration of protein in saline only. Mice that were immunised in this way received 140 μg of Der p 1 in total, compared with 24 μg Der p 1 administered to the

alum mice. Although seven times as much protein had to be administered, the results were extremely encouraging.

It is difficult to directly compare local draining lymph node responses between the two protocols, as the same lymph nodes were not examined. It was thought that the lymph nodes closest to the site of intranasal (cervical)/intratracheal (tracheal) immunisation should be examined. Consequently, local immune responses from alum-treated mice were associated with the upper airways, whilst the adjuvant-free mice were associated with the lower airways. A strong *in vitro* recall response was also detected from tracheal lymphocytes but in contrast, there was minimal response from the cervical lymphocytes of the Der p 1/alum treated mice (figure 3.1). This may reflect differences in the amount of protein administered (60 μg:20 μg). Also, intranasal administration of a single dose of Der p 1 which was used in the alum treated mice may have failed to provide sufficient antigen to recall responses in the draining lymph nodes due to limited uptake. However, with intratracheal instillation, which was the mode of delivery in the adjuvant-free protocol, all of the antigen would remain within the lung and therefore, enter the immune system.

From the tracheal lymph node culture supernatants, we were able to detect the presence of IL-5 (figure 3.2) and IL-13 (figure 3.3), which were both induced in an antigen-dependent manner. No IFN-γ was detected which suggests that the local cytokine environment was predominantly of the Th2 phenotype. From these results we can see that the cytokines tested from the cervical lymph node cultures, taken from the Der p 1/alum treated mice were below the threshold for detection. This is hardly surprising when proliferation levels were so low.

Despite receiving less protein intranasally, the Der p 1/alum mice still exhibited high levels of eosinophilia that were certainly comparable to the adjuvant-free mice (figure 3.6). This observation suggests that sufficient antigen enters the lung to recruit eosinophils although is not capable of driving a recall response in the draining lymph nodes. Cytospins prepared from the BAL fluid of the adjuvant-free mice support the previous results, revealing visually the extent of lung eosinophilia. Control mice that had been systemically immunised with Der p 1 in the same manner but had received only saline intratracheally showed no eosinophilia with the predominant cell type being macrophages (figure 3.7). Although we were unable to

detect IL-5 or IL-13 in the cultures prepared from cervical lymph nodes, both IL-5 and IL-4 were detected in BAL fluid taken from the Der p 1 in alum treated mice (figures 3.4 and 3.5). It is difficult to explain why IL-5 and IL-13 could not be detected in the Der p 1 in alum cervical lymph node cultures. The only explanation that can be given is that the supernatants from these mice deteriorated before the presence of these cytokines was established. If there had been more time available and a more plentiful supply of Der p 1, it would have been preferable to repeat the experiment again in order to confirm and optimise the conditions.

Th2 responses were not investigated in the spleens from any mice because we wished to focus on local responses in the airways, particularly pulmonary eosinophilia. However, later experiments in which the adjuvant-free protocol has been adopted, a dose dependent proliferative response of splenocytes in response to Der p 1 is observed (counts usually in the range 15,000-20,000 CPM at 10 μg/ml Der p 1). This degree of responsiveness was comparable to that observed for the Der p 1/alum immunised mice (results not shown).

The results presented here suggest that Der p 1/alum induces the most marked production of antigen-specific antibodies (figure 3.8). This is not unexpected due to the depot effect of the alum on releasing the Der p 1, nevertheless moderate antibody levels were still observed in the adjuvant-free immunised mice. The predominance of Der p 1-specific IgG1 further substantiates previous findings suggesting the presence of a Th2 response.

In conclusion, we have shown that an allergic Th2 response with accompanying lung inflammation, can be induced without the use of alum adjuvant.

Chapter 4

Microencapsulated Particles

4.1 Introduction

New approaches to vaccine development include the synthesis of protein or peptide sequences, which are homologous with epitopes capable of inducing protective immunity against infectious organisms or innocuous allergens. This 'subunit approach' has a number of advantages over more traditional vaccines; they are chemically well defined, and can be prepared reproducibly and inexpensively. However, these subunit vaccines often display poor immunogenicity, resulting in the need for repeated immunisations. One approach to overcome these problems involves the use of controlled antigen delivery systems, which includes the encapsulation of antigen [476].

Most microencapsulated vaccine systems consist of poly(lactide-co-glycolide) (PLG) polymers containing the antigen [448, 449], virus [450] or peptide [451] of interest. PLG microparticles are suitable vehicles for vaccine delivery because they are biocompatible and biodegradable. PLG has had a history of safe use in humans since the 1970s where the material has been used as resorbable sutures [452]. Microparticles only induce a minimal inflammatory response after immunisation and degrade to the normal metabolites – lactic acid and glycolic acid by non-enzymic hydrolysis [453].

The particles have the ability to release entrapped antigen over extended periods in a pulsatile manner depending on the ratios of lactic acid and glycolic acid. The fastest degradation rates are observed with microparticles consisting of a 50:50 ratio. These are eliminated from the body in 50 – 60 days after subcutaneous or intramuscular injection with a degradation half-life in the order of 14 days. Increasing the lactic acid leads to extended retention in the circulation because it is

more hydrophobic in nature than glycolic acid and therefore release rates can be extended up to one year [454].

Another benefit for the use of microparticles is that a single injection of mixed ratio particles, can induce an immune response that mimics traditional multiple dosing schedules [455]. For example, a single immunisation with diphtheria toxoid (DT) entrapped in controlled release microparticles can induce comparable immune responses to three doses of DT adsorbed to alum [456]. In addition, microparticles containing peptide or protein immunogens have been reported to induce HIV neutralising antibodies for at least one year following a single immunisation [457].

Particle size is an important parameter for determining immunogenicity. For parenteral vaccination, the particles should be of a size that allows uptake by macrophages and transport to the lymphatics. Only particles less than 10 μm are taken up in this way [458].

The induction of mucosal immune responses by oral delivery of microparticles is highly desirable; advantages include the non-invasive protocol, cost effectiveness and formulations for oral delivery could stabilise the vaccines making them highly appropriate for use in third world countries. There are also important immunological reasons for delivering vaccines by this route. Most human pathogens gain access to their host across a mucosal surface. The gastrointestinal tract is largest mucosal surface in man, so immune responses mounted at these surfaces would provide a first line of defence, limiting or even preventing pathogens from attaching to and crossing the mucosa [459].

PLG microparticles are particularly suitable for oral delivery because the polymer is not broken down by enzymic degradation or acid hydrolysis in the gut and thus the entrapped antigen remains protected. The first studies using PLG to encapsulate proteins as subunit vaccines appeared in 1989 [460]. This work showed successful elicitation of circulatory anti-toxin antibodies and also specific secretory IgA antibodies in saliva, gut and broncho-alveolar washes, following oral immunisation of PLG encapsulated Staphylococcal enterotoxin B (SEB) toxoid. No immune response was detected when non-encapsulated SEB was administered orally. Thus microencapsulation had both a protective and immunopotentiating effect,

probably resulting from a depot effect of antigen and also the direct delivery of relatively high antigen concentrations into accessory and antigen-presenting cells [461, 462]

Again, uptake of particles was restricted by size, only those with a diameter <10 μm were taken up into the Peyer's Patches. Those with diameters 5-10 μm, were retained for extended periods whilst particles <5 μm were rapidly redistributed to the spleen.

A priority area for future research would be to improve the uptake of microparticles following oral administration. It is thought that only a tiny fraction (<1%) of ingested microparticles are taken up by the gut-associated lymphoid tissue (GALT) [463]. Therefore, specific improvements in targeting vaccines to the mucosae may result in the enhancement of an immune response.

The adsorption of antigen (tetanus toxoid) onto poly(L-lactic acid) microparticles as opposed to encapsulation has also been shown to have an effect on the immune response, when administered intranasally [464]. Higher antibody responses were induced with adsorbed tetanus toxoid as opposed to free antigen.

Microparticles can also be used to induce cytotoxic T lymphocyte (CTL) responses in a number of models. Early studies in mice show that oral or intraperitoneal administration of microparticles containing ovalbumin, induced CTL responses in the spleen and draining lymph nodes *in vitro* [465, 466]. Later studies associated with disease models have also shown CTL induction. For example, the CTL epitope of the measles virus was encapsulated and after a single i.p. injection, CTL responses to the homologous peptide and measles virus were detected over a period of four months. Responses peaked at 30 days after priming and were maintained at high levels for 120 days. The responses were higher than those observed when the CTL epitope was administered in saline or as an emulsion in Incomplete Freund's Adjuvant [451, 467]. A CTL response has also been observed by encapsulating a synthetic malaria epitope and injecting the particles i.p. or s.c.. Mice that received two immunisations produced 6- to 8-fold higher activity and 5- to 40-fold higher antibody responses than those mice that only received one injection [468]. This finding suggests that a memory immune response was induced with microparticle formulations.

Therefore, from the evidence above, it is clear that PLG microparticles are capable of eliciting strong antibody, T cell proliferative and CTL responses. However, the mechanisms of processing and presentation of microencapsulated antigens and their fate is still poorly understood. A recent study [469] has attempted to investigate the capacity of human or murine macrophages and other APCs to process and present microencapsulated antigens to MHC class I or class II-restricted T cells *in vitro*. It was demonstrated that macrophages were able to present exogenous microencapsulated antigen to both MHC class I- and class II-restricted T cells *in vitro* and this was thought to occur principally by size dependent phagocytosis. In addition, immature dendritic cells have also been shown to be phagocytic [470]. It is possible that the upregulation of dendritic cells *in situ* (specifically the Langerhans cells), plays an important role in the uptake of PLG encapsulated antigen and subsequent migration to the draining lymph nodes, where DCs can efficiently prime T cell responses.

In conclusion, microencapsulation of antigens might be of great importance in developing subunit vaccines against bacterial, viral and parasital infections or tumours where both humoral and cellular immunity are crucial.

4.2 Visualisation of MEA Particles

ALK, the sponsors of this study developed histological techniques for the visualisation of MEA particles. Figures 4.1- 4.3 represent studies undertaken in the sponsor's laboratory in Denmark. The figures have been included in this thesis purely to demonstrate how the MEA particles can be visualised *in vitro* and *in vivo*.

Figure 4.1 shows light microscopy of PLG particles loaded with fluorescent allergen in a water suspension. The allergen used in this study was an aqueous allergen extract derived from the pollen of timothy grass, *Phleum pratense*. Freeze dried extract of *Phleum pratense* was conjugated with Fluorescein isothiocyanate (FITC). PLG microparticles were then loaded with the FITC-*Phleum pratense* conjugate using the method described by Tice [471]. The microparticles were suspended in water, spread on microscope slides, allowed to air dry and visualised at the following magnifications: a: 40x, b: 63x and c: 100x. The labelled allergen appears green and is located within the PLG particles.

Figure 4.2 represents an immunohistochemical method for detecting allergen within the particles. Lung tissue (figure 4.2b) from mice immunised intravenously with MEA particles was treated and prepared for paraffin embedding, sectioned, deparaffinated and immunohistochemistry performed by incubating sections with monoclonal mouse antibodies to *Phleum pratense*. The particles were also visualised *in vitro* by light microscopy (figure 4.2a).

Figures 4.3a to 4.3f show immunohistochemistry of *Phleum pratense* microparticles in suspension (a and b), in lung tissue (c and d) and in kidney tissue (e and f). Figures. 4.3a, c, and e represent positive controls of immunised mice, whereas, figures. 4.3b, d, and f represent negative controls from unimmunised animals. In the positive controls allergen was clearly visible in the centre and along the periphery of the particles as visualised by reddish-brown dots. In the negative microparticle suspension control (4.3b) only a very weak outline of particles was seen. In the tissue sections the particles were spread evenly in both lung and kidney tissues. A positive immune reaction was observed in the positive controls (c and e), depicted by long arrows. Additionally a non-specific but distinct immunoreaction was observed in some of the nuclei of the cells in the lung and kidney sections (positive and negative controls), which is depicted by short arrows.

Fig. 4.1a

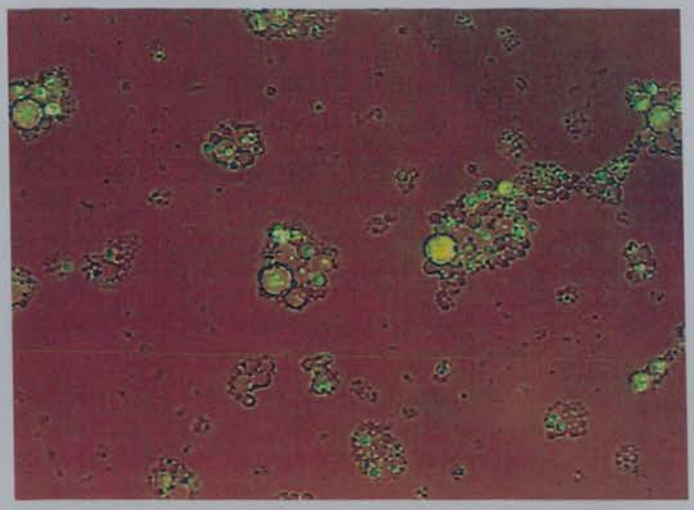


Fig. 4.1b

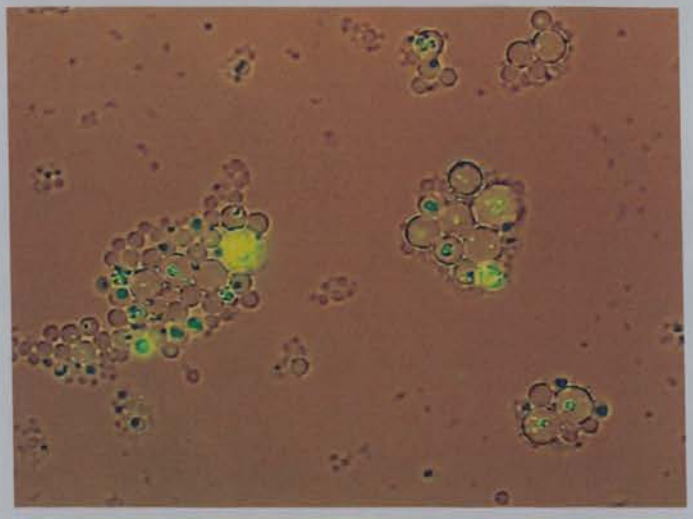


Fig. 4.1c

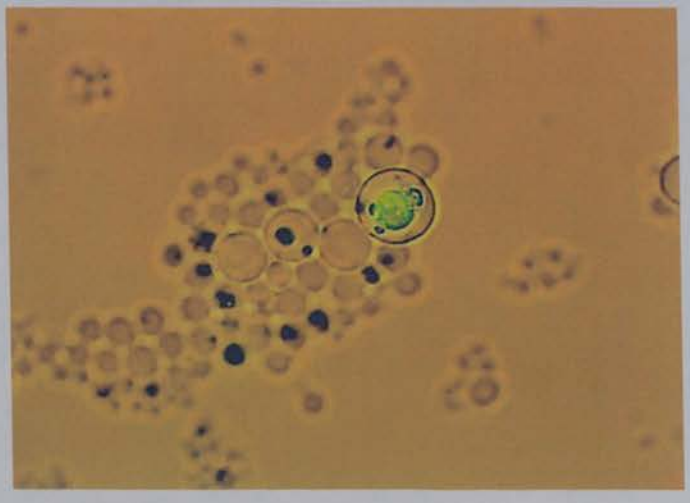


Figure. 4.1. Visualisation of MEA particles.
Mix of light- and fluorescence microscopy of the PLG particles loaded with FITC labelled allergen.
The objectives used are a: 40x, b: 63x and c: 100x. The labelled allergen appears green and is located within the PLG particles.

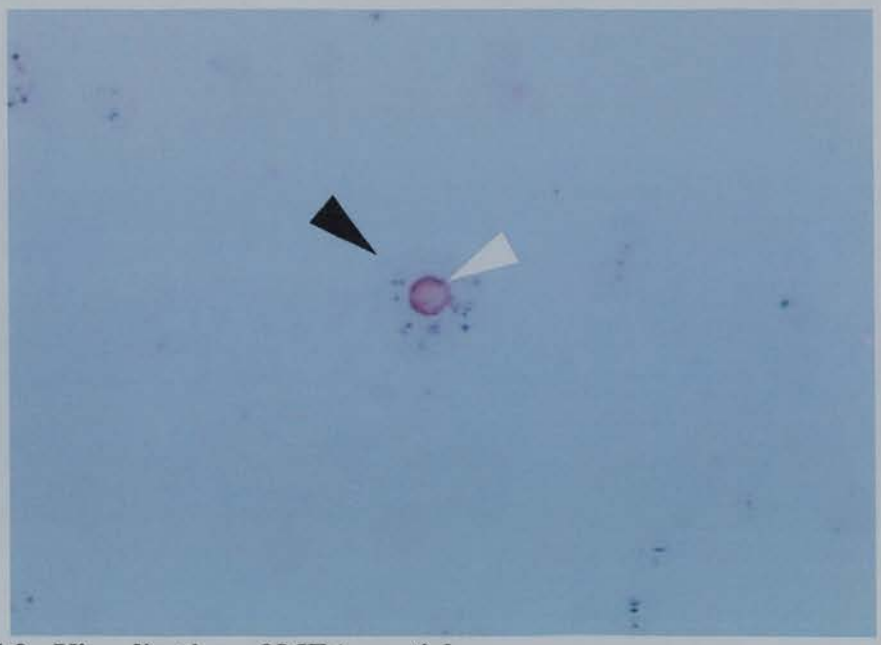


Figure 4.2a Visualisation of MEA particles.
Light microscopy of a particle using a 100X objective.
Immunohistochemistry has been performed on the particle suspension. A reaction (red colour, white arrow) is clearly seen in the aggregate of allergens in the centre of the particle. The black arrow shows the outline of the particle.

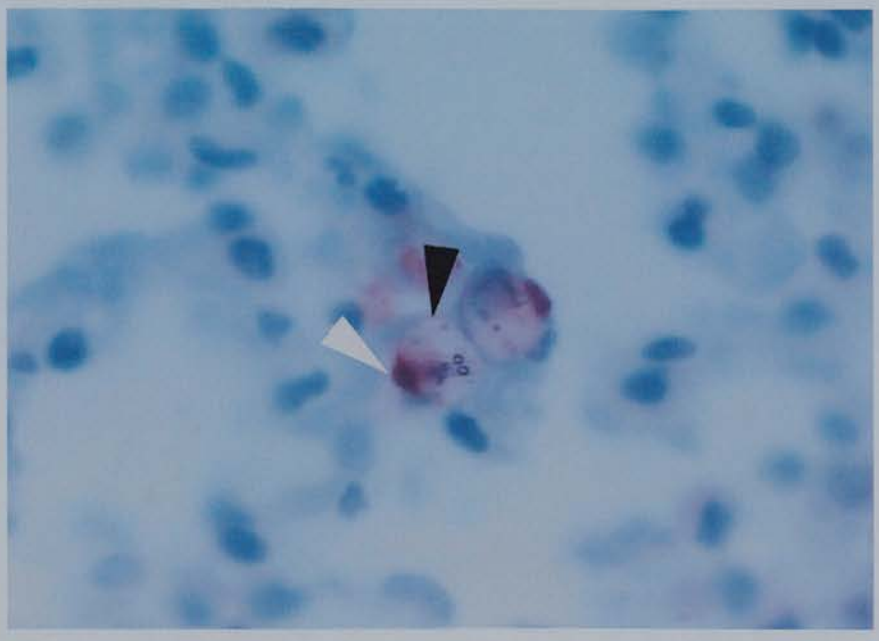


Figure 4.2b Visualisation of MEA particles.
Lung tissue from a mouse given MEA particles intravenously (objective used 100x).
In the section, the allergens show a positive immune reaction (white arrow) within the particles (black arrow). The reaction here is located in the periphery of the particle.

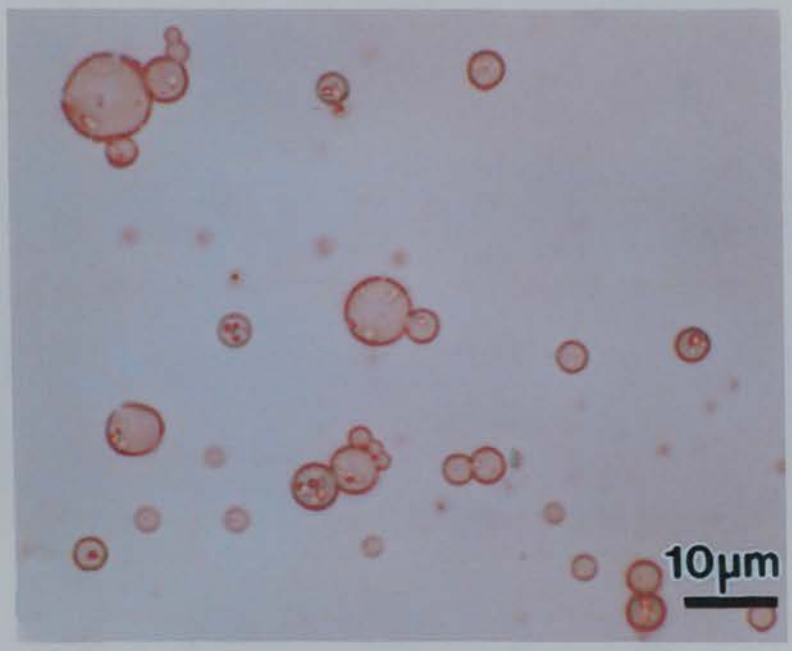


Figure 4.3a Visualisation of MEA particles.

Positive immune reaction towards a Phleum pratense suspension of microparticles.
The immune reaction is present in the centre and along the periphery of the particles. Magnification 900x.

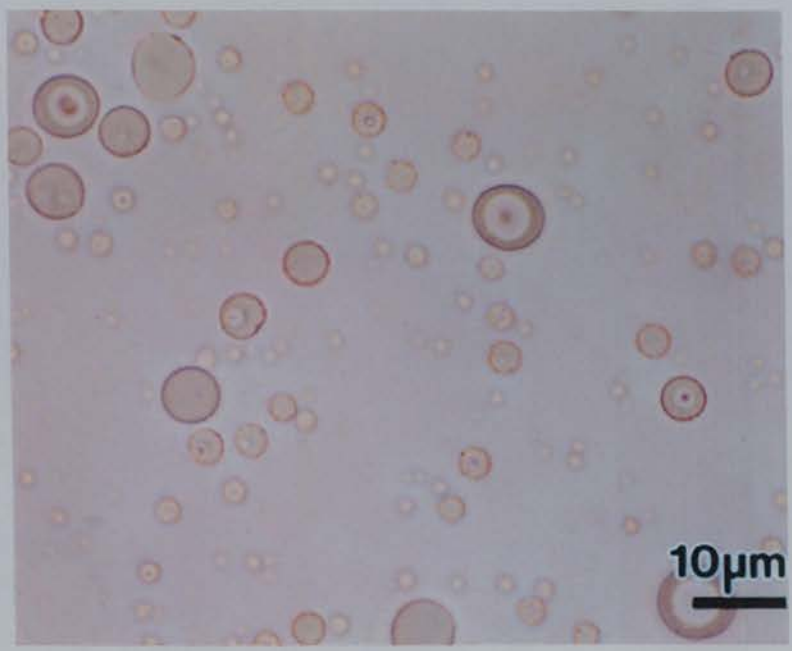


Figure 4.3b Visualisation of MEA particles.
Negative immune reaction towards a Phleum pratense suspension of microparticles.
No reaction is seen and the particles are outlined by a very weak pale red colour. Magnification 900x.

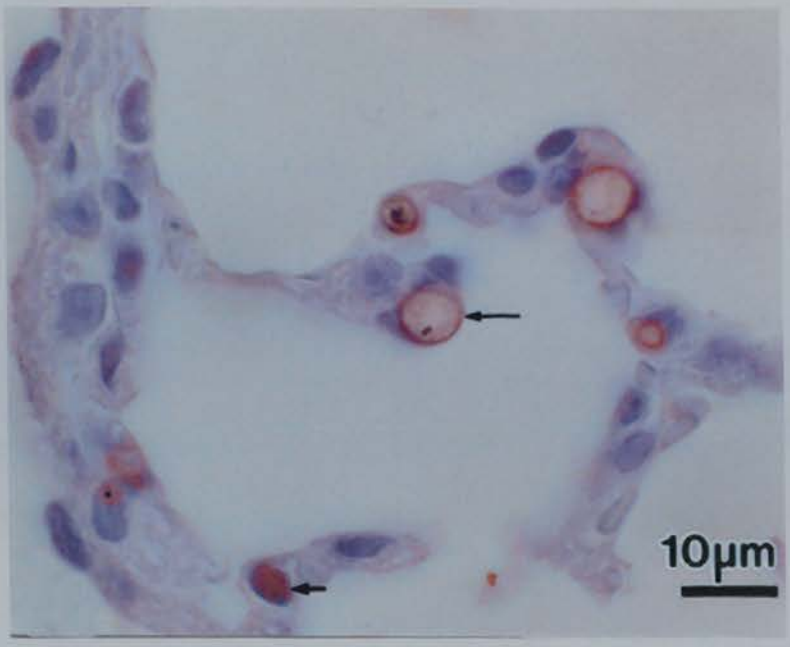


Figure 4.3c Visualisation of MEA particles.
Tissue sample from the lung of an intravenously-immunised mouse.
A clear immune reaction is present in the particles in the tissue (shown by long arrows). Magnification 900x.

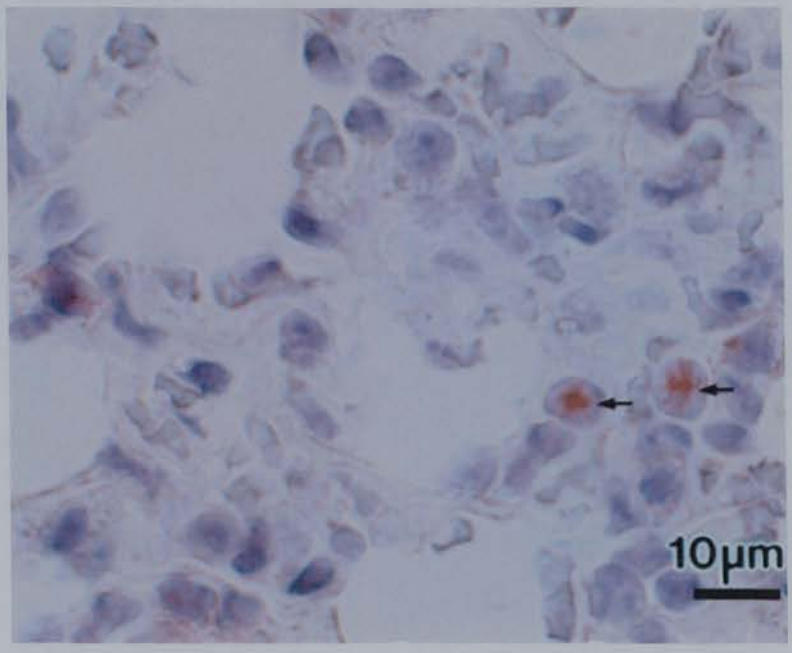


Figure 4.3d Visualisation of MEA particles.
Lung section from an unimmunised control mouse.
Secondary AEC staining (from the substrate 3-amino-9-ethylarbazole) of the nuclei (short arrows) was observed in d as well as e. Magnification 900x.

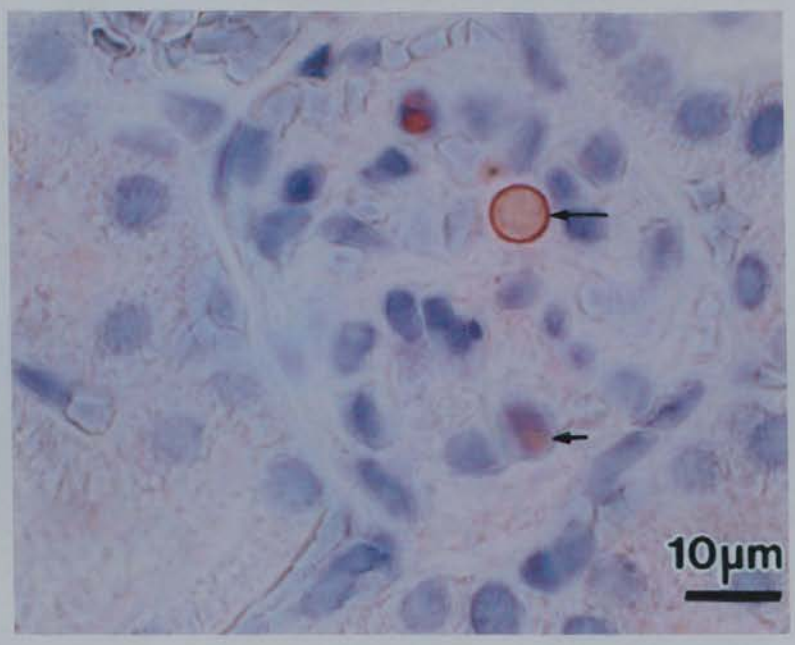


Figure 4.3e Visualisation of MEA particles.
Tissue sample from the kidney of an intravenously-immunised mouse.
A clear immune reaction is present in the particles in the tissue (shown by long arrows). Magnification 900x.

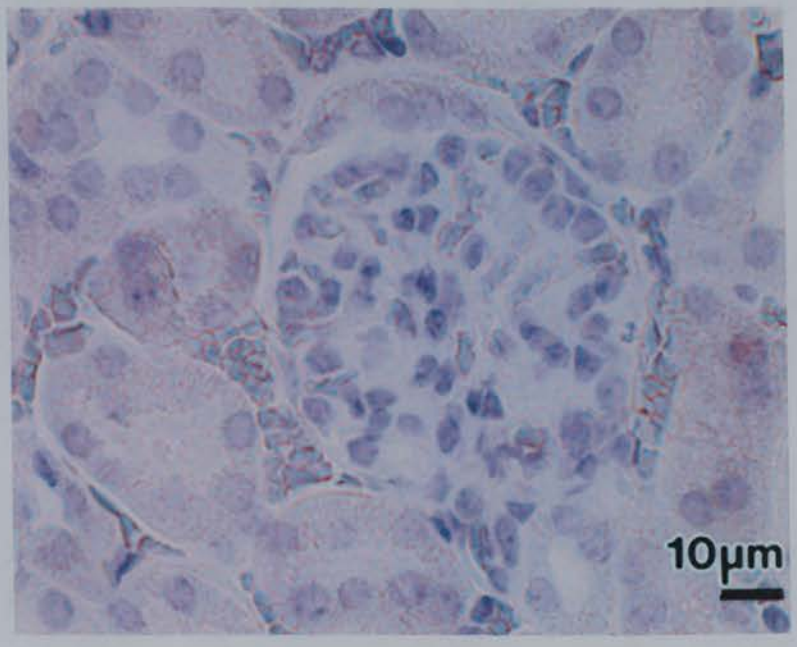


Figure 4.3f Visualisation of MEA particles.
Kidney section taken from an unimmunised control mouse.
Magnification is 600x.

Results

Immunisation of unsensitised mice with MEA Der p 1

4.3 Induction of proliferative, cytokine and antibody responses after sub-cutaneous, intra-peritoneal and intra-venous immunisation with MEA Der p 1.

The objective of these experiments was to establish the kinetics and determine the effect of MEA Der p 1 when administered via different immunisation routes, in the absence of adjuvant. Naïve female H-2^b (C57BL/6) mice were immunised with 7 mg MEA Der p 1 microcapsules which contained approximately 50 μg Der p 1. The mice were immunised on day 0 either subcutaneously (s.c.) (n=5), intraperitoneally (i.p.) (n=5) or intravenously (i.v.) (n=5). The spleens, draining lymph nodes and sera were removed either 9, 21 or 42 days later. These time points were chosen because antigen specific memory responses would be present in the spleen by day 9 and the aim of this study was to investigate whether or not a significant response would still be detectable 21 and 42 days after immunisation. Splenocytes were cultured in complete media either alone or in the presence of 0.2, 2 or 20 μg/ml Der p 1. Unless otherwise stated, the following graphs represent mean values from pooled data obtained from individual mice.

Figure 4.4 shows splenocyte proliferation at days 9, 21 and 42. Each graph represents the dose-responsive proliferation to Der p 1 in the three groups of mice following different routes of immunisation (s.c., i.p. and i.v.). Immunisation with MEA Der p 1 by each of the chosen routes induces marked priming, however, there is little difference between each group of mice at the three time points.

For the draining lymph nodes (para-aortic for i.p., inguinal for s.c. and both para-aortic and inguinal for i.v.) there were insufficient cells from the i.p. and the i.v. groups of mice to set up proliferation assays so only data from the s.c. group is shown (figure 4.5). The lymphocytes were cultured in complete media alone or in the

presence of 0.2, 2 and 20 μg/ml of the immunodominant peptide of Der p 1, residues 111-139. The highest level of proliferation was observed at day 9. By day 21, levels had decreased and by day 42 there was no antigen-specific proliferation. IFN-γ and IL-5 were not detected in any of the lymphocyte culture supernatants. The threshold of sensitivity of the ELISAs (IFN-γ and IL-5) was in the region of 0.2 ng/ml.

In contrast, IFN-γ could be detected in some of the splenocyte culture supernatants. The highest levels of IFN-γ were observed in the day 9 splenocyte cultures (figure 4.6). Differences in the route of immunisation were evident, with the i.p. route exhibiting the highest levels of IFN-γ in response to restimulation with 2 and 20 μg/ml Der p 1. Levels of IFN-γ were diminished by day 21 and were virtually non-existent by day 42. There was no detectable IL-5 in any of the splenocyte cultures (results not shown).

Figure 4.7 shows the levels of Der p 1-specific IgG1, IgG2a and total IgE from the sera of the mice at the three time points. The sera was titrated at three concentrations (1:10, 1:100 and 1:1000) with results shown for the 1:100 dilution. As expected, Der p 1-specific IgG1 was barely detectable in any of the mice at day 9. However, levels had markedly increased by day 21 and were higher still at day 42. There was little to differentiate between the groups of mice at day 21 and it was hard to compare the different routes at day 42 because the i.v. sera was only diluted 1 in 10 as opposed to 1 in 100. This was due to experimental error. There was insufficient sera to retest at limiting dilutions.

Levels of Der p 1-specific IgG2a and total IgE were observed but these were below 5 ng/ml and were far reduced compared to IgG1.

At this time, we had not developed an ELISA for Der p 1-specific IgE so only total IgE was measured. There is barely any difference in total IgE levels when comparing days 9 and 21, with only a slight increase at day 42 (figure 4.7). Overall, total IgE levels were not elevated to the same extent as Der p 1-specific IgG1 but IgE levels are always lower than IgG.

IL-2 (supernatants removed at 24 hours from splenocyte cultures) and IL-4 (supernatants removed at 48 hours from splenocyte cultures) levels were also

measured using IL-2 and IL-4 dependent cell lines (HT-2 and CT.4S respectively). Neither cytokine was detectable in any of the culture supernatants.

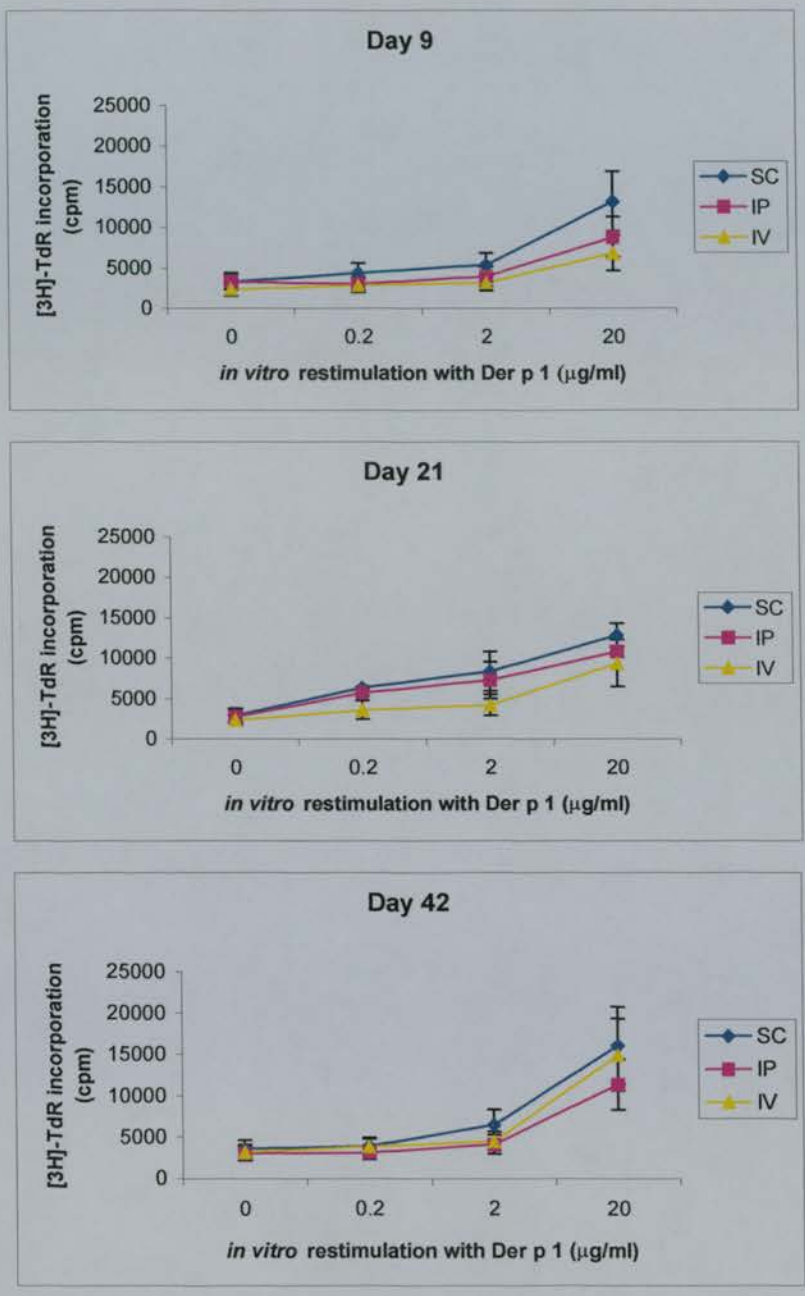


Figure 4.4. Immunisation with MEA Der p 1 via different routes.
Days 9, 21 and 42 - Proliferation of splenocytes.
C57BL/6 mice were immunised with 7 mg MEA Der p 1 either s.c. (n=5), i.p. (n=5) or i.v. (n=4). The spleens were removed either 9, 21 or 42 days later and cultured with media alone or in the presence of 0.2, 2 or 20 µg/ml Der p 1. Proliferation was measured at 72 hours after pulsing with thymidine at 66 hours.
Mean +/- SE is shown.

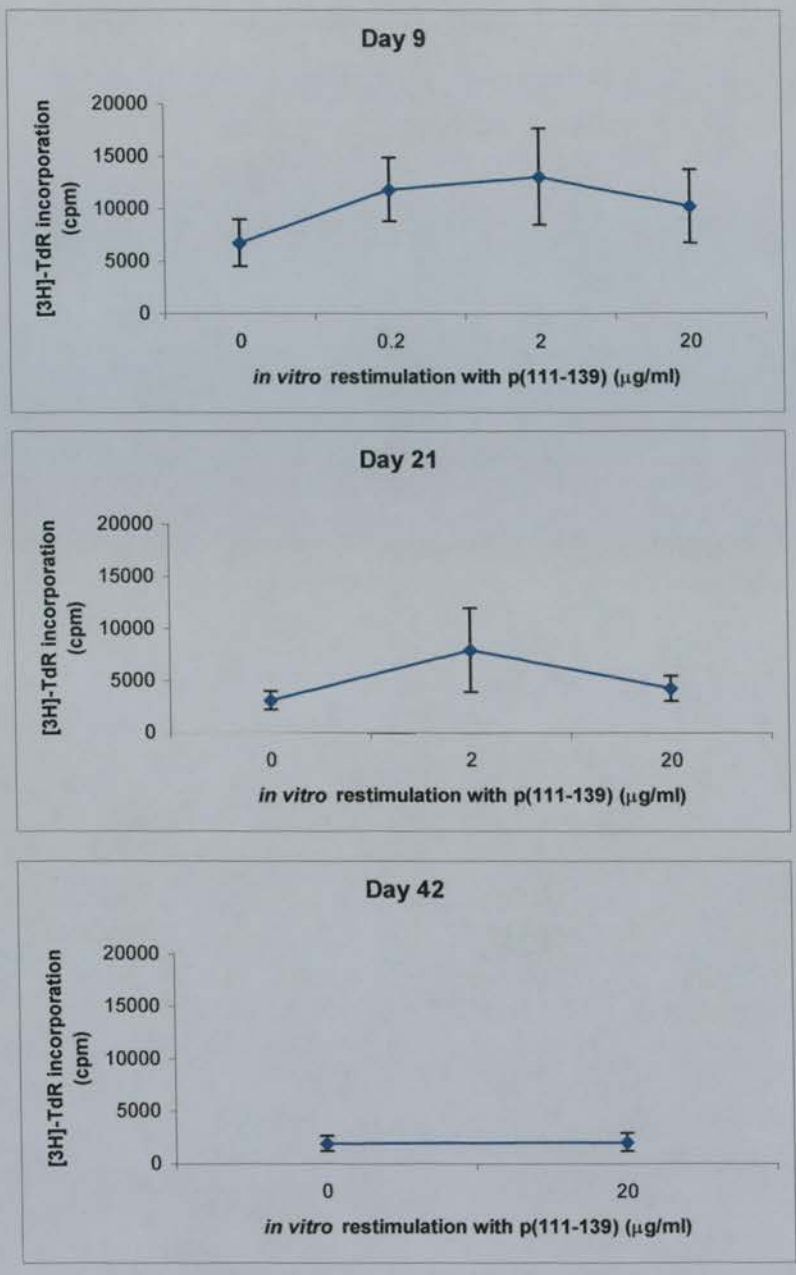


Figure 4.5. Immunisation with MEA Der p 1 via different routes. Proliferation of lymphocytes from the inguinal lymph nodes of the sub-cutaneously immunised mice.

C57BL/6 mice were immunised with 7mg MEA Der p 1 either s.c. (n=5), i.p. (n=5) or i.v. (n=4). The draining lymph nodes were removed either 9, 21 or 42 days later and cultured with media alone or in the presence of 0.2, 2 or 20µg/ml p(111-139). Proliferation was measured at 72 hours after pulsing with thymidine at 66 hours. There were insufficient cells to set up cultures for the i.p. and i.v. mice. Mean +/- SE is shown.

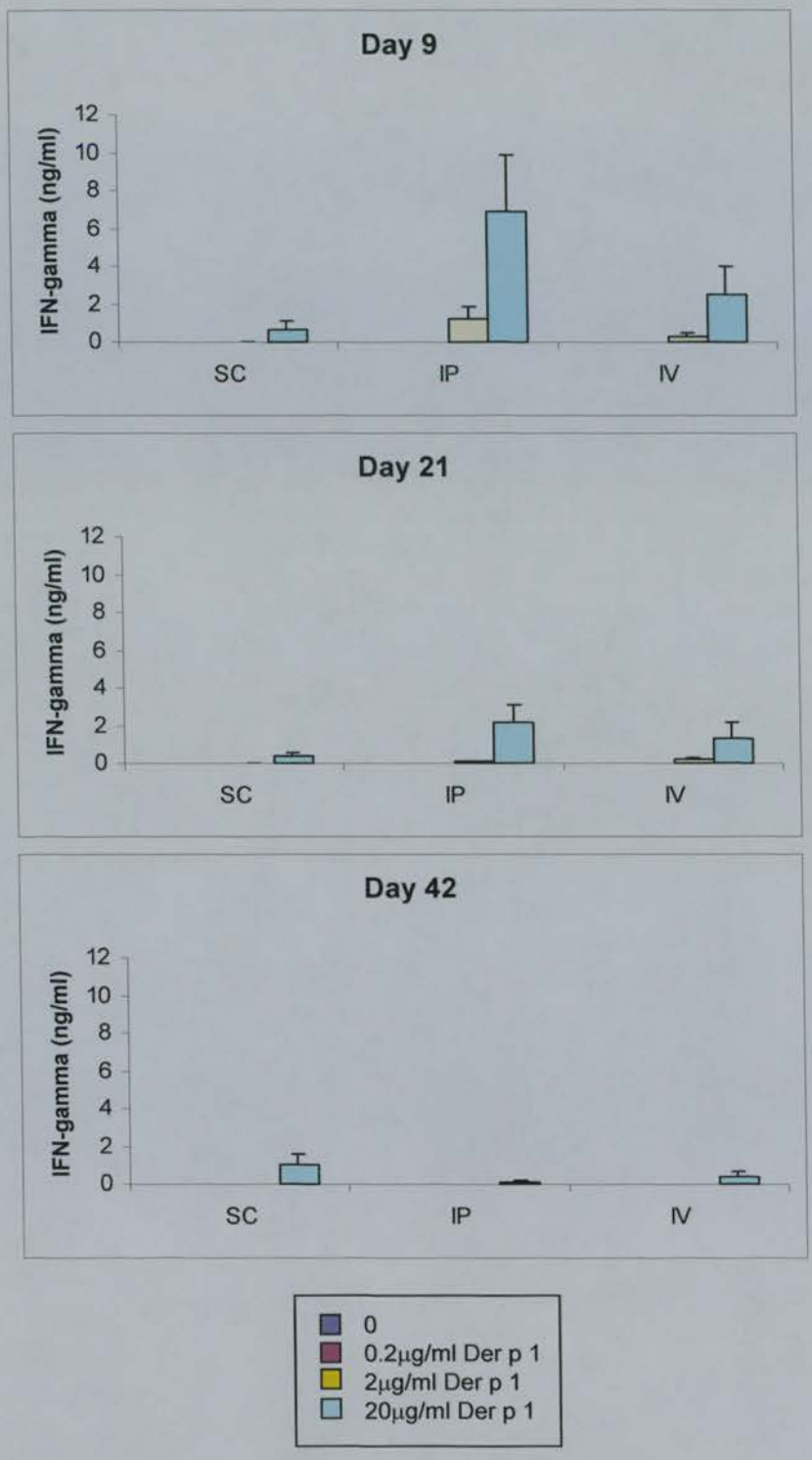


Figure. 4.6. Immunisation with MEA Der p 1 via different routes.
Days 9, 21 and 42 – IFN-γ production by splenocytes.
Splenocytes (4 x 10^5/well) were cultured in media alone or in the presence of 0.2, 2 or 20 μg/ml Der p 1. Supernatants were removed at 48 hours and frozen at -20°C until analysis by ELISA.
Mean +/- SE is shown

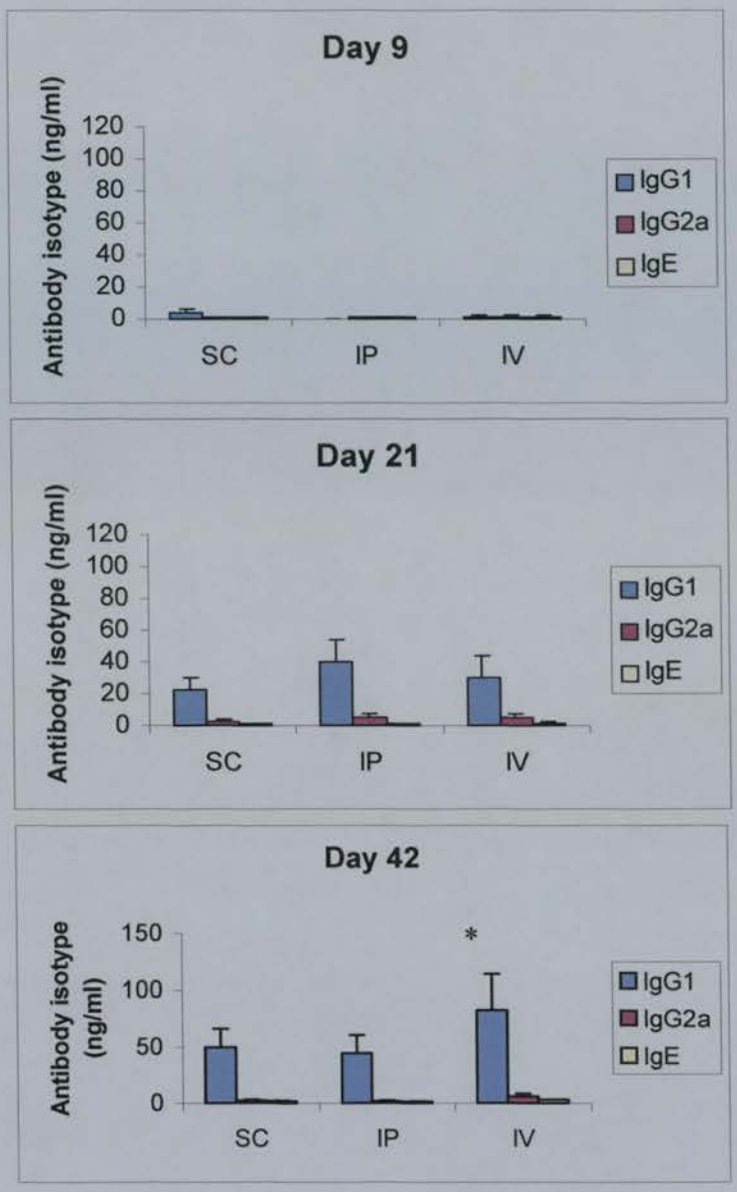


Figure 4.7. Immunisation with MEA Der p 1 via different routes. Der p 1 – specific IgG1, IgG2a and total IgE levels in sera of mice sacrificed at 9, 21 and 42 days.
The presence of antibodies was detected by ELISA.
The sera was diluted 1:100.
* Sera diluted 1:10.
Mean +/- SE is shown.

4.4 Sub-cutaneous immunisation with varying amounts of Der p 1

After establishing the basic kinetics to induce maximum T cell and B cell responses with subcutaneous, intraperitoneal and intravenous immunisation, it was decided to investigate the subcutaneous and intraperitoneal (discussed later) routes of immunisation in more depth because these routes had exhibited stronger immune responses than the intravenous route. In these experiments, the effect of dosage on the immune response was measured. Firstly, subcutaneous delivery was analysed. In these experiments, the amount of microencapsulated (MEA) Der p 1 was varied and placebo microparticles (containing no Der p 1) were included in the study along with a positive control - Der p 1 in Complete Freunds' Adjuvant (CFA).

Naïve female C57BL/6 mice were immunised with either 7 mg, 14 mg or 30 mg of MEA Der p 1 microcapsules (containing 50 μg, 100 μg or 150 μg Der p 1 respectively) or 7 mg placebo microcapsules (n=5 for each group of mice) or 50 μg Der p 1 in CFA. The mice were immunised s.c. on day 0 and sacrificed 11 days later, when spleens, inguinal lymph nodes and sera were removed for analysis. Unless otherwise stated, the following graphs represent mean values from pooled data obtained from individual mice (n=5). The experiment was repeated twice with the strongest results being shown.

Figure 4.8 shows splenocyte proliferation. Mice immunised with MEA Der p 1 all exhibited dose-responsive proliferation but there was no marked difference between the groups. The levels of proliferation were actually slightly elevated to that induced with Der p 1in CFA.

Figure 4.8 also shows lymphocyte proliferation from the inguinal lymph node cultures. A small background response was observed in mice immunised with placebo particles, suggesting that the PLG polymer of the microparticles induced a small amount of non-specific proliferation. The 7 mg and 30 mg MEA Der p 1 groups showed maximal proliferation at the 2 μg dose of Der p 1;p111-139, which decreased at 20 μg/ml Der p 1;p111-139. P111-139 has been shown to be an immunodominant epitope of Der p 1 in mice on a H-2^b background [560]. Again

proliferation levels for the MEA Der p 1 groups were higher than the Der p 1/CFA group.

IFN-γ levels in the supernatants obtained from placebo splenocyte cultures were barely detectable (figure 4.9). IFN-γ production was evident in all the MEA Der p 1 mice. Maximal IFN-γ levels were observed in the 14 mg group; and at a restimulation concentration of 20 μg/ml, this was more than two-fold greater than that produced by the splenocytes from the 7 mg MEA Der p 1 group. IFN-γ production declines by approximately 30% in the 30 mg MEA Der p 1 mice. The 7 mg group was comparable to that induced with Der p 1/CFA. No IFN-γ was detected in any of the lymph node cultures.

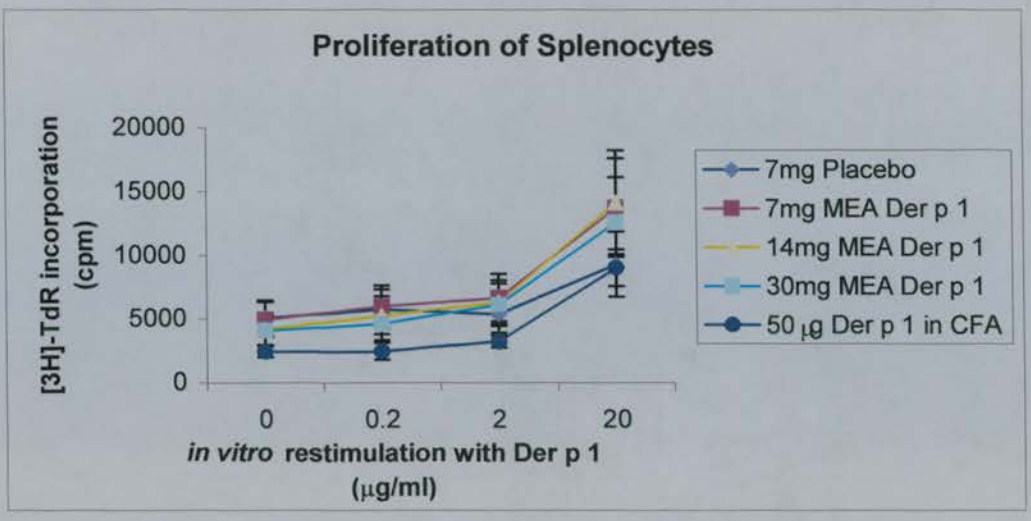
There was no detectable IL-5 in any of the splenocyte or lymph node cell cultures.

The highest levels of Der p 1-specific IgG1 were found in the Der p 1/CFA group which is somewhat surprising as the administration of CFA is usually associated with Th1 responses and thus production of IgG2a, however, there were marked levels of Der p 1-specific IgG1 in the three groups of mice immunised with MEA Der p 1 (figure 4.10). The highest levels were observed in the 30 mg group. Placebo-immunised mice had Der p 1-specific IgG1 levels barely above background.

All groups exhibited small levels of Der p 1-specific IgG2a (figure 4.10), with the 14 mg group exhibiting slightly higher levels than the rest.

Der p 1-specific IgE was determined for all mice with the exception of the Der p 1/CFA group. Production of IgE was dose responsive with respect to MEA dosage. Although a clear trend can be observed, this was not statistically significant.

In summary, maximum proliferation was observed in the 7 mg MEA Der p 1 immunised mice. Maximum cytokine production was observed in the 14 mg MEA Der p 1 mice with maximum antibody responses being observed in the 30 mg MEA Der p 1 mice. The reductions in proliferation and cytokine production in the 30 mg MEA Der p 1 mice compared to the 7 mg MEA Der p 1 mice suggest that high doses of MEA may be detrimental. However, as the placebo particles were only tested at a dose of 7 mg, we cannot be certain that this is the case.



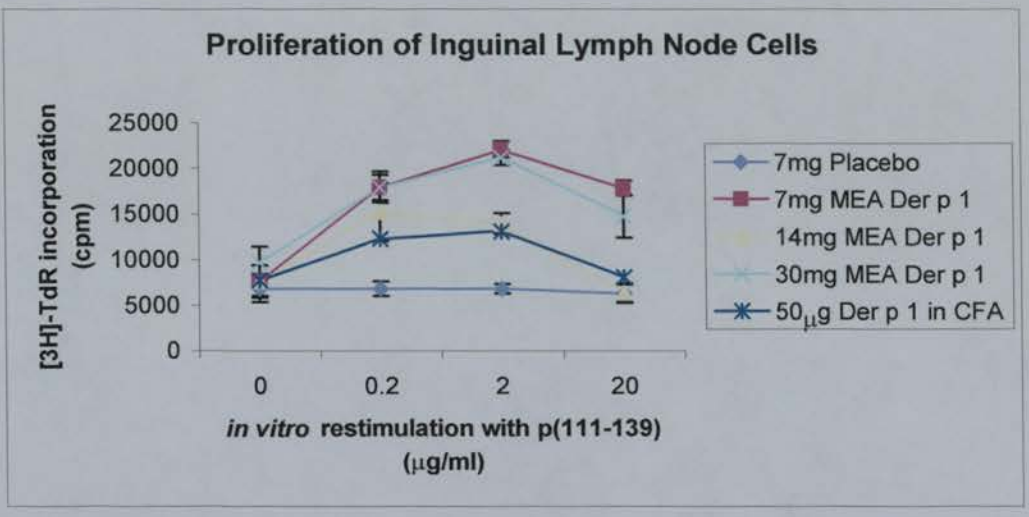
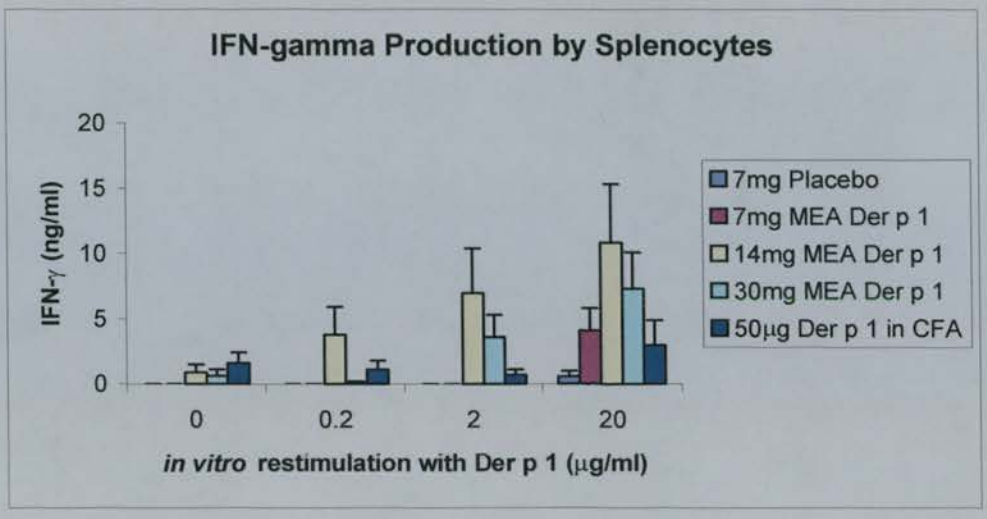


Figure 4.8. Subcutaneous with varying amounts of MEA Der p 1. Proliferation of splenocytes and lymphocytes.
C57BL/6 mice were immunised with either 7 mg placebo, 7 mg, 14 mg or 30 mg of MEA Der p 1. The spleens and inguinal lymph nodes were removed 11 days later. Single cell suspensions (4 x 10^5/well splenocytes, 2 x 10^5/well inguinal LN cells) were cultured with media alone or in the presence of 0.2, 2 or 20 μg/ml Der p 1 (for splenocytes) or Der p 1;p111-139 (for inguinal LN cells).
Mean +/- SE is shown.



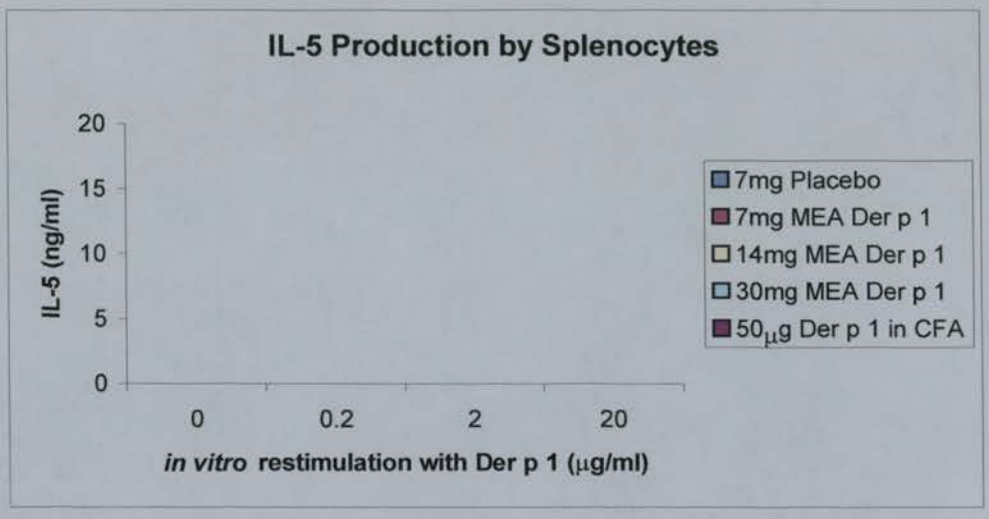
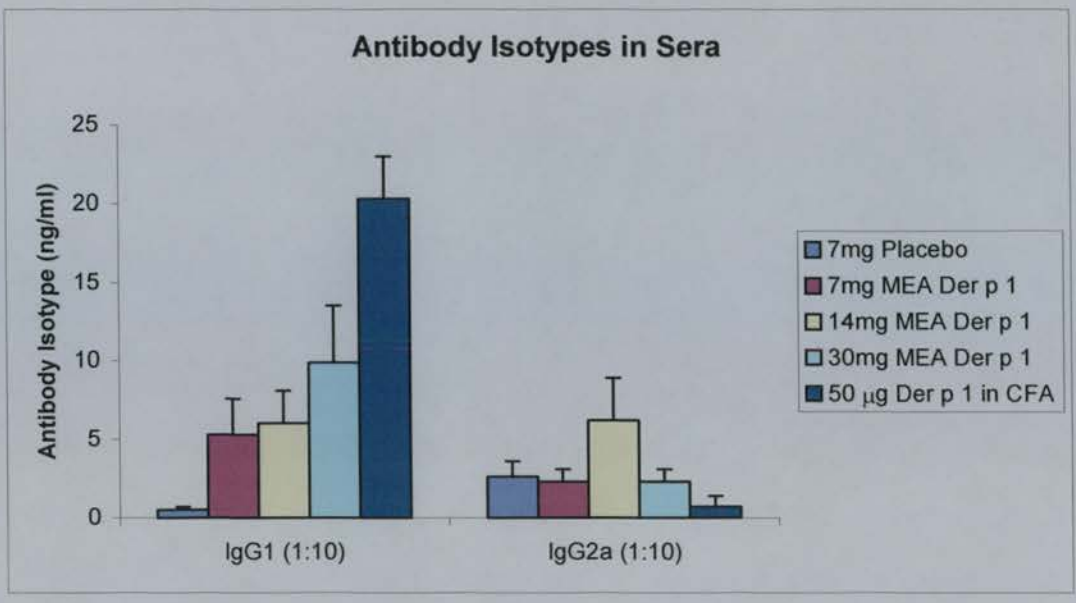


Figure 4.9. Subcutaneous immunisation with varying amounts of MEA Der p 1. IFN-γ and IL-5 levels in splenocyte supernatants.
Splenocytes (4 x 10^5/well) were cultured in media alone or in the presence of 0.2, 2 or 20 μg/ml Der p 1. Supernatants were removed at 48 hours and frozen at -20°C until analysis by ELISA.
Mean +/- SE is shown.



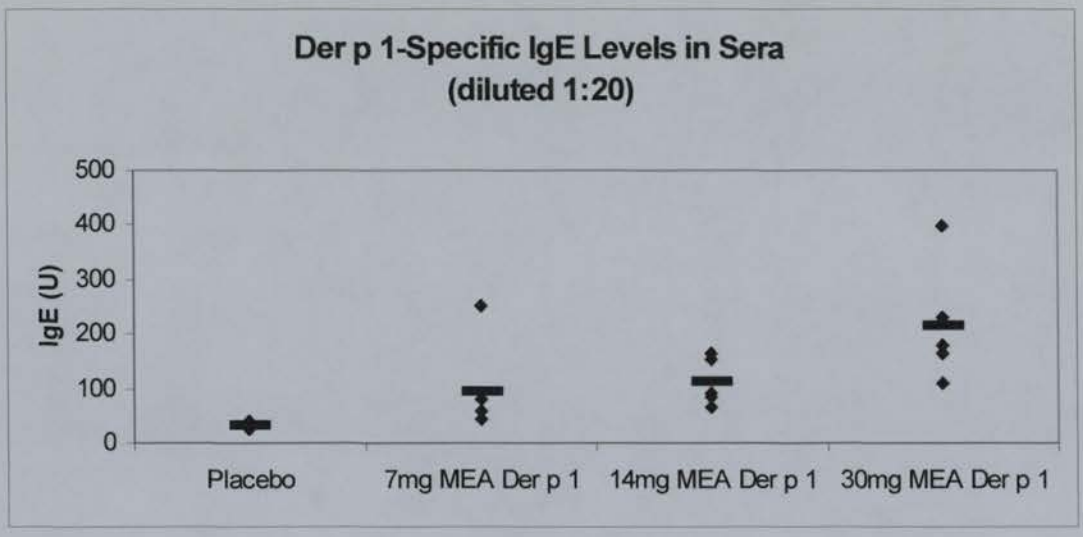


Figure 4.10. Sub-cutaneous immunisation with varying amounts of MEA Der p 1.
Der p 1-specific IgG1, IgG2a and IgE levels in sera.
Antibody levels were detected by ELISA.
Sera was diluted 1:10 for IgG1 and IgG2a. Sera was diluted 1:20 for IgE. There was insufficient sera to test IgE levels in Der p 1/CFA mice.
Mean +/- SE is shown for upper chart.

4.5 Intra-peritoneal immunisation with varying amounts of Der p 1

The aim of these experiments was to determine the optimal dosage of MEA Der p 1 given intraperitoneally to mice. A placebo group was not included in this study due to a limited supply of mice and materials. In addition, it was felt that the effects of placebo microparticles had already been determined in previous experiments.

Naïve female C57BL/6 mice were immunised with either 3.5 mg, 7 mg, 14 mg or 30 mg of MEA Der p 1 microcapsules (containing approximately 25 μg, 50 μg, 100 μg or 150 μg Der p 1). The mice were immunised i.p. on day 0 and sacrificed 21 days later, when spleens, para-aortic lymph nodes (PALN) and sera were removed for analysis.

Figure 4.11 shows the levels of splenocyte and lymphocyte proliferation in response to restimulation with Der p 1 and Der p1;p111-139 respectively. Control polyclonal stimuli of α-CD3 and α-CD28 were also set up but results have not been shown. All groups of mice show dose-dependent proliferation but there is little to distinguish between the groups. Proliferation of the para-aortic lymph nodes barely rises above background levels.

IFN-γ was detectable in all of the splenocyte culture supernatants and was dose-dependent with regards to MEA Der p 1 dosage; however, no IL-5 was detected in these culture supernatants (figure 4.12). The level of IFN-γ produced was approximately 20 % of that produced by the subcutaneous route of administration. However, this may be explained by kinetics. Intraperitoneally immunised mice were sacrificed at day 21, whereas, subcutaneously immunised mice were sacrificed at day 11.

The lymph node culture supernatants produced no detectable IFN-γ or IL-5 which can probably be attributed to cell numbers (2×10^5/well).

Der p 1-specific IgG1 was only detectable in the 30 mg MEA Der p 1 group (figure 4.13). This was comparable to the levels observed for the 30 mg MEA Der p 1 s.c.- immunised mice (figure 4.10). There was no detectable Der p 1-specific IgG2a in any of the groups. Der p 1-specific IgE was detected in all three groups but there

was no significant difference between experimental groups. Levels of IgE observed were only slightly greater than the sensitivity of the ELISA (20 U) (figure 4.13).

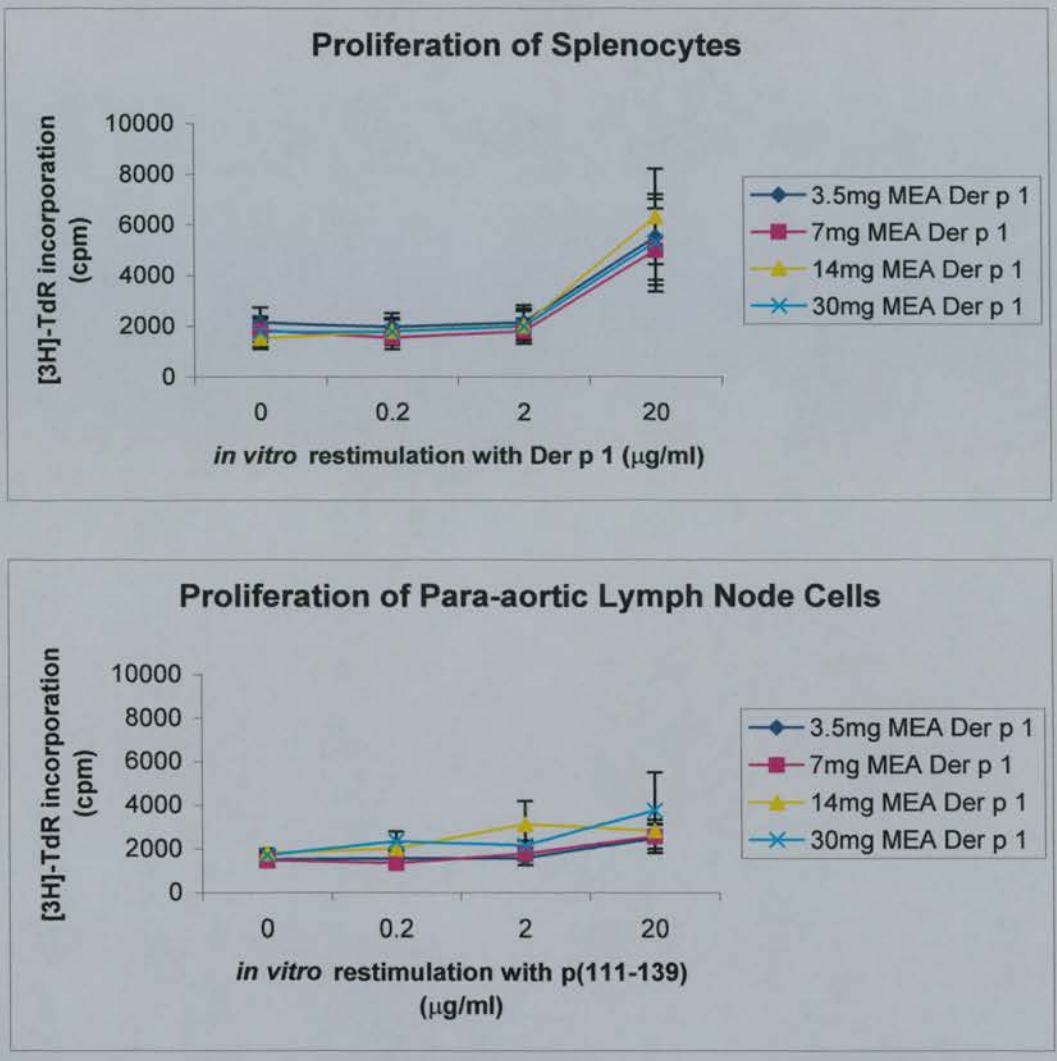
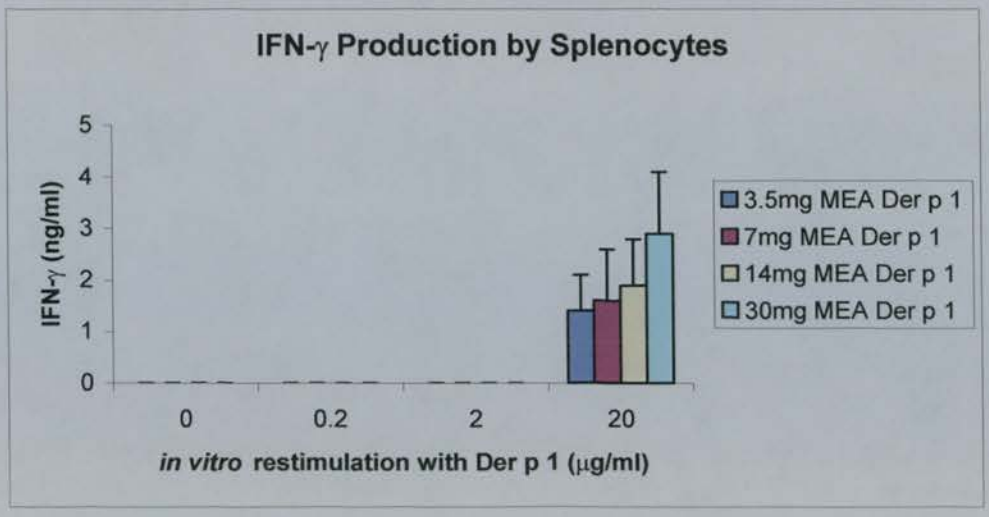


Figure 4.11. Intra-peritoneal immunisation with varying amounts of MEA Der p 1.
Proliferation of splenocytes and para-aortic lymph node cells.
C57BL/6 mice were immunised with either 3.5 mg, 7 mg, 14 mg or 30 mg of MEA Der p 1. The spleens and para-aortic lymph nodes were removed 21 days later. Single cell suspensions (4 x 10^5/well splenocytes, 2 x 10^5/well para-aortic lymph node cells) were cultured with media alone or in the presence of 0.2, 2 or 20 μg/ml Der p 1 (for splenocytes) or Der p 1;p111-139 (for PALN cells).
Mean +/- SE is shown.



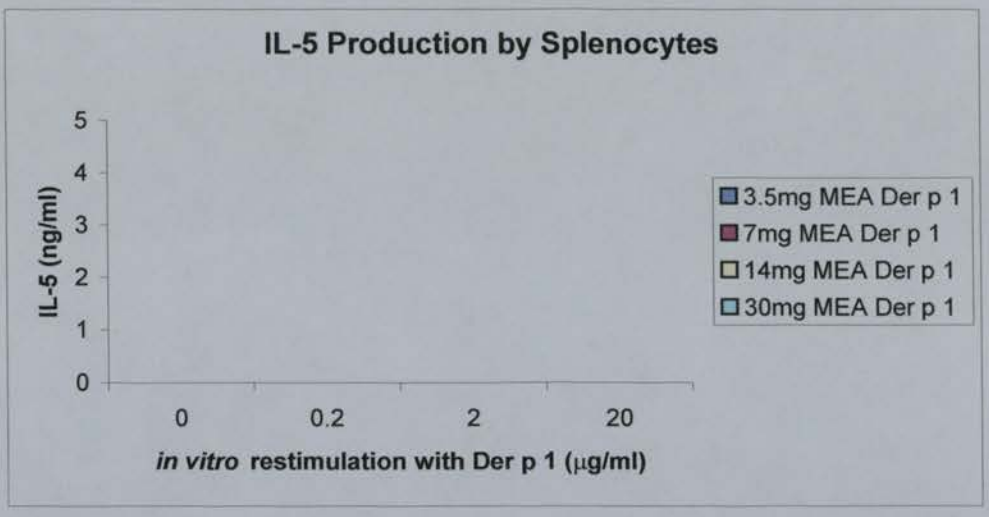
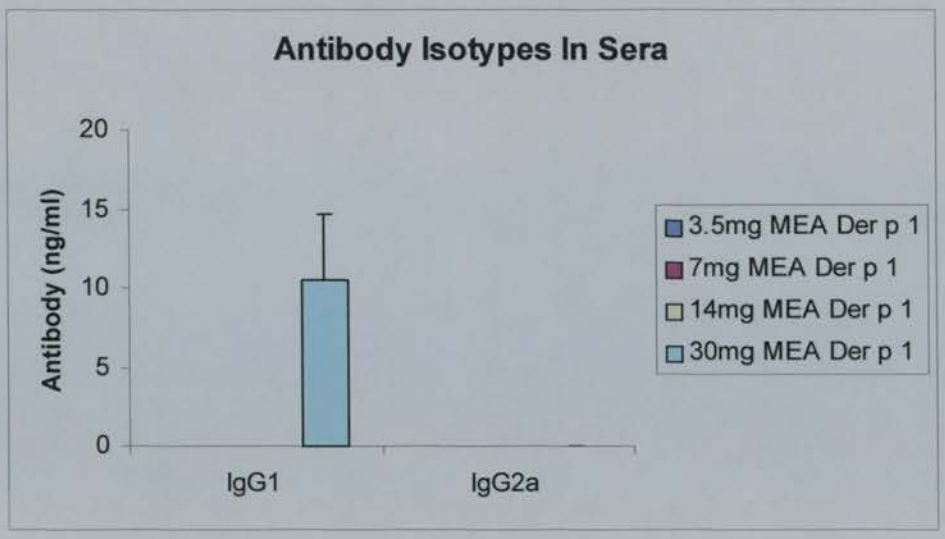


Figure 4.12. Intra-peritoneal immunisation with varying amounts of MEA Der p 1.
IFN-γ and IL-5 levels production by splenocyte supernatants.
Splenocytes (4 x 10^5/well) were cultured in media alone or in the presence of 0.2, 2 or 20 μg/ml Der p 1. Supernatants were removed at 48 hours and frozen at -20°C until analysis by ELISA.
Mean +/- SE is shown.



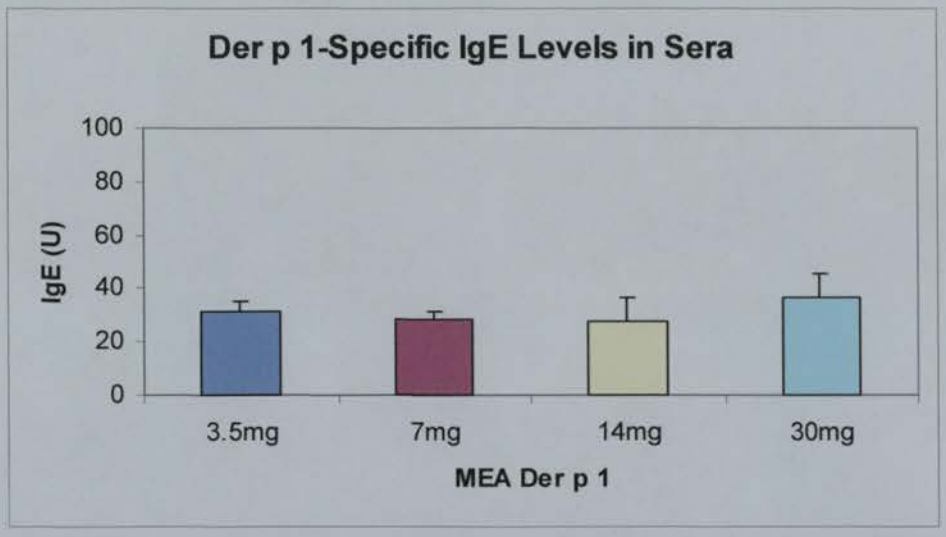


Figure 4.13. Intra-peritoneal immunisation with varying amounts of MEA Der p 1.
Der p 1-specific IgG1, IgG2a and IgE levels in sera.
Antibody levels were detected by ELISA.
Sera was diluted 1:10 for IgG1 and IgG2a. Sera was diluted 1:20 for IgE.
Mean +/- SE is shown.

4.6 Oral Administration of MEA Der p 1

A number of studies have shown that the oral administration of microparticles containing antigen induces systemic responses [466, 472]. We decided to investigate whether the oral administration of MEA Der p 1 could also prime antigen-specific responses. Previous studies using three consecutive oral doses of MEA Der p 1 (126 mg MEA Der p 1 containing 900 μg Der p 1 in total) hardly induced an immune response (results not shown), combined with a study by Lavelle, suggesting that only a tiny fraction (<1%) of ingested microparticles were actually taken up by the gut-associated lymphoid tissue [463], a multiple-dosing schedule was adopted, in an attempt to ensure that sufficient protein was administered.

Five C57BL/6 female mice were fed by oral gavage with 30 mg of MEA Der p 1 in 200μl 0.87% NaCl. Before the gavage, the mice were lightly anaesthetised with halothane gas. The mice were gavaged on five consecutive days and then rested for two days. This procedure was repeated twice more so that the mice received a total of 450 mg MEA Der p 1, which contained approximately 3.2 mg Der p 1. Another five mice received the same amount of placebo particles. Although this seems a very large dose of antigen to administer, previous studies using ovalbumin as the model antigen use a similar amount (3 mg) in their immunisation protocols [465, 472].

Unfortunately, a control group of Der p 1 in saline was not included in this study because of difficulties in obtaining sufficient quantities of antigen. All mice were sacrificed eleven days after the final immunisation when the spleen, mesenteric lymph nodes, Peyer's patches and sera were removed.

Figure 4.14 shows splenocyte, lymph node and Peyer's patch proliferation respectively from both the MEA Der p 1 and placebo mice. There was a small amount of splenocyte proliferation in both MEA Der p 1 and placebo treated mice at a restimulation concentration of 20 μg/ml Der p 1. This suggests that there may have been traces of endotoxin present in the purified Der p 1. Splenocytes and mesenteric lymph node cells from both groups of mice proliferated well in response to concanavalin A (used as a positive control), proving that MEA Der p1 was not toxic

to these cells (figure 4.15). However, there was a marked decrease in proliferation of the Peyer's patch cell cultures from the MEA Der p 1 mice compared with placebo, suggesting that there the Peyer's patch cells were more sensitive to any potential toxic effects of MEA Der p 1.

IFN-γ and IL-5 levels were not detected in either the spleen, mesenteric lymph node or Peyer's patch cell cultures.

Figure 4.16 shows the Der p 1-specific IgG1 and IgG2a levels in sera. A very small amount (<0.5 ng/ml) of IgG1 was detected in both groups of mice but this is on the threshold of the ELISA's sensitivity. No Der p 1-specific IgG2a was detected in either group of mice.

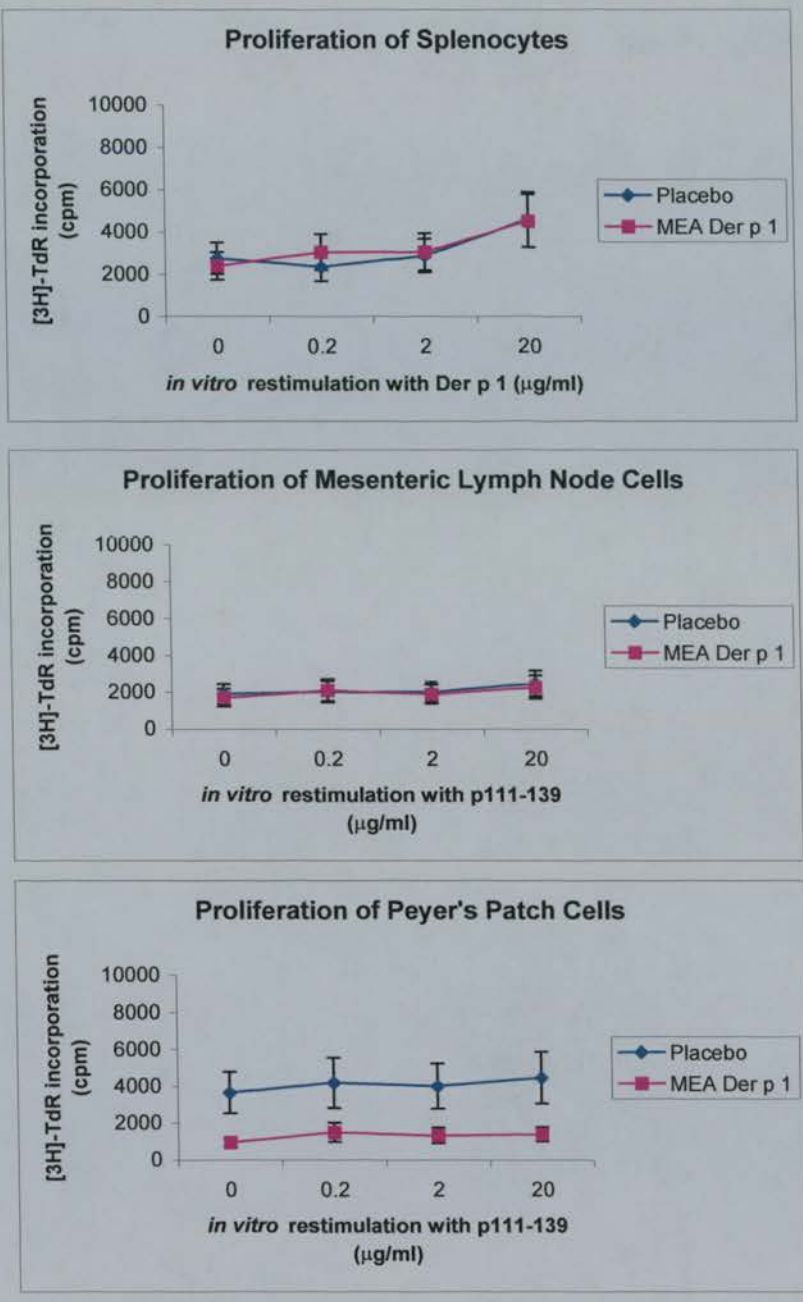


Figure 4.14. Oral administration with placebo or MEA Der p 1. Proliferation of splenocytes, MLN cells and Peyer's patch cells.
C57BL/6 mice were given placebo or MEA Der p 1 by oral gavage. The spleens, mesenteric lymph nodes and Peyer's patches were removed 11 days after the final administration. Single cell suspensions (4×10^5/well splenocytes, 2×10^5/well lymphocytes and Peyer's patch cells) were cultured with media alone or in the presence of 0.2, 2 or 20 µg/ml Der p 1 (splenocytes) or Der p 1;111-139 (lymphocytes and Peyer's patch cells). Proliferation was measured at 72 hours after pulsing with thymidine at 66 hours.
Mean +/- SE is shown.

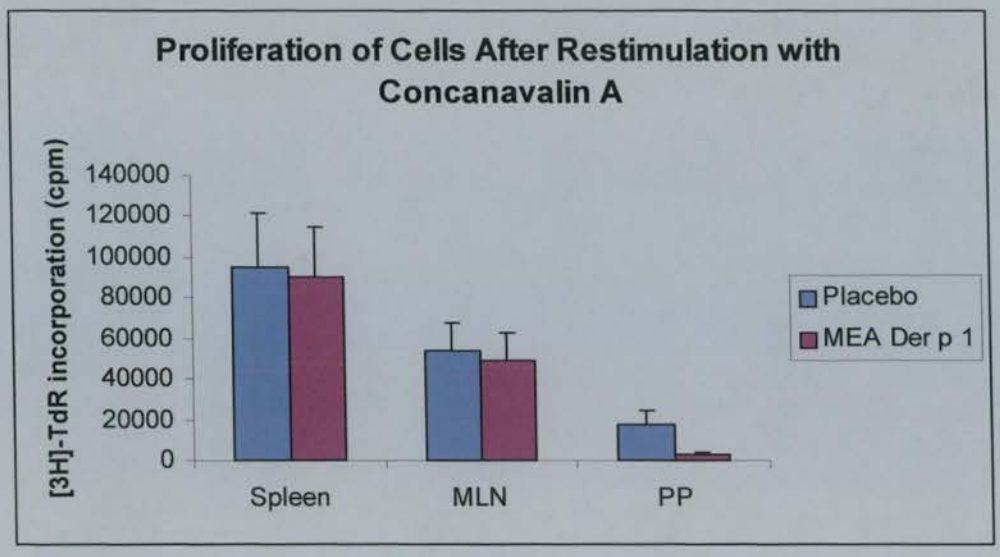


Figure 4.15. Oral administration with placebo or MEA Der p 1. Proliferation of splenocytes, MLN and Peyer's patch cells in response to concanavalin A.

C57BL/6 mice were given placebo or MEA Der p 1 by oral gavage. The spleens, mesenteric lymph nodes and Peyer's patches were removed 11 days after the final administration. Single cell suspensions (4 x 10^5/well splenocytes, 2 x 10^5/well MLN and Peyer's patch cells) were cultured with 1 μg/ml concanavalin A. Proliferation was measured at 72 hours after pulsing with thymidine at 66 hours.
Mean +/- SE is shown.

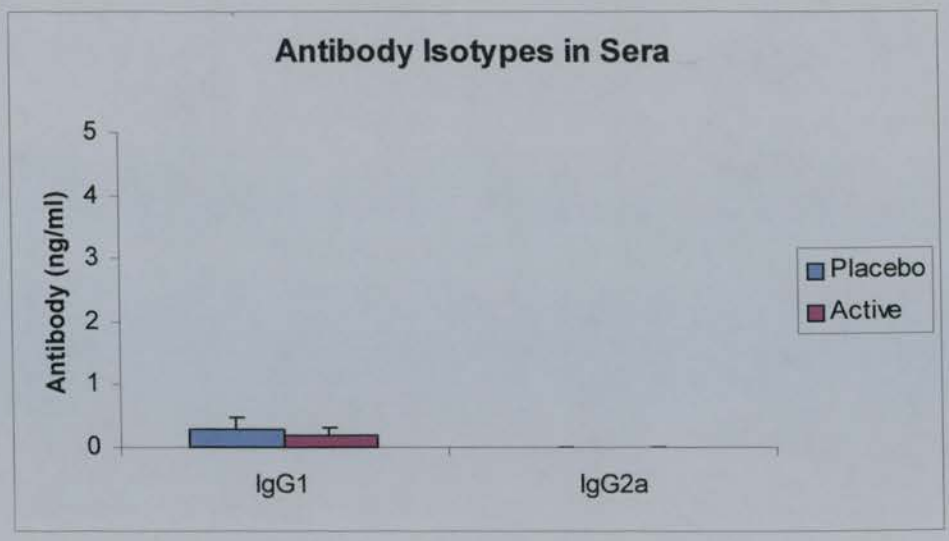


Figure 4.16. Oral administration with placebo or MEA Der p 1. Der p 1-specific IgG1 and IgG2a levels in sera.
Antibody levels were detected by ELISA.
Sera was diluted 1:10.
Mean +/- SE is shown.

4.7 Assessment of Tolerance Induction by Oral Feeding in the Mouse

The objective of this study was to assess whether oral administration of MEA Der p 1 induced tolerance. The findings of the previous experiment discussed in this chapter suggested that the lack of an immune response could be attributed to tolerance induction but from the immunisation protocol used it was impossible to determine this. Two groups of mice were included in this study, one group was fed with 42 mg MEA Der p 1 comprising 300 μg Der p1, on three consecutive days with a control group receiving the same amount of placebo microparticles. Thirty three days after the last oral administration, all mice received 50 μg Der p 1 in CFA. After a further ten days the mice were sacrificed and spleen, inguinal lymph nodes and sera were removed. This protocol is different to the one adopted in the previous experiment because the amount of MEA Der p 1 available was a limiting factor. However, preliminary experiments had shown that a weak immune response could be induced with three oral immunisations of 42 mg of MEA Der p 1 (results not shown). Therefore, due to material constraints, it was decided to apply this knowledge to determine whether oral administration of MEA Der p 1 resulted in tolerance induction.

Figure 4.17 shows the splenocyte and inguinal lymph node proliferation levels in response to Der p 1 and Der p 1;p111-139 respectively. Both groups of mice exhibited equal levels of splenocyte proliferation at the highest dose of Der p 1 (20 μg/ml). The proliferation of inguinal lymph node cells peaked at 2 μg/ml Der p 1;p111-139 and was three times higher in the MEA Der p 1 mice (although SE values are large).

IFN-γ was detected in the splenocyte culture supernatants from the MEA Der p 1 mice only (figure 4.18). The highest level observed was with the 20 μg/ml of Der p 1. It was a little strange that no detectable IFN-γ was observed in splenocyte cultures from the placebo mice after having received an immunisation of Der p1 in CFA. This may be attributed to the sensitivity of the ELISA which had a sensitivity threshold in the region of < 500 pg/ml. IL-5 was not detected in any of the culture supernatants.

Der p 1-specific IgG1 and IgG2a were not detected in any of the mice but total IgE and small amounts of antigen-specific IgE were detected (figure 4.19). However, there were no significant differences between the two groups of mice. Levels of Der p 1-specific IgE were just above the sensitivity of the ELISA (20 U) but the fact that the placebo particles induce Der p 1-specific IgE suggests that the Der p 1 in CFA is inducing a small amount of IgE production.

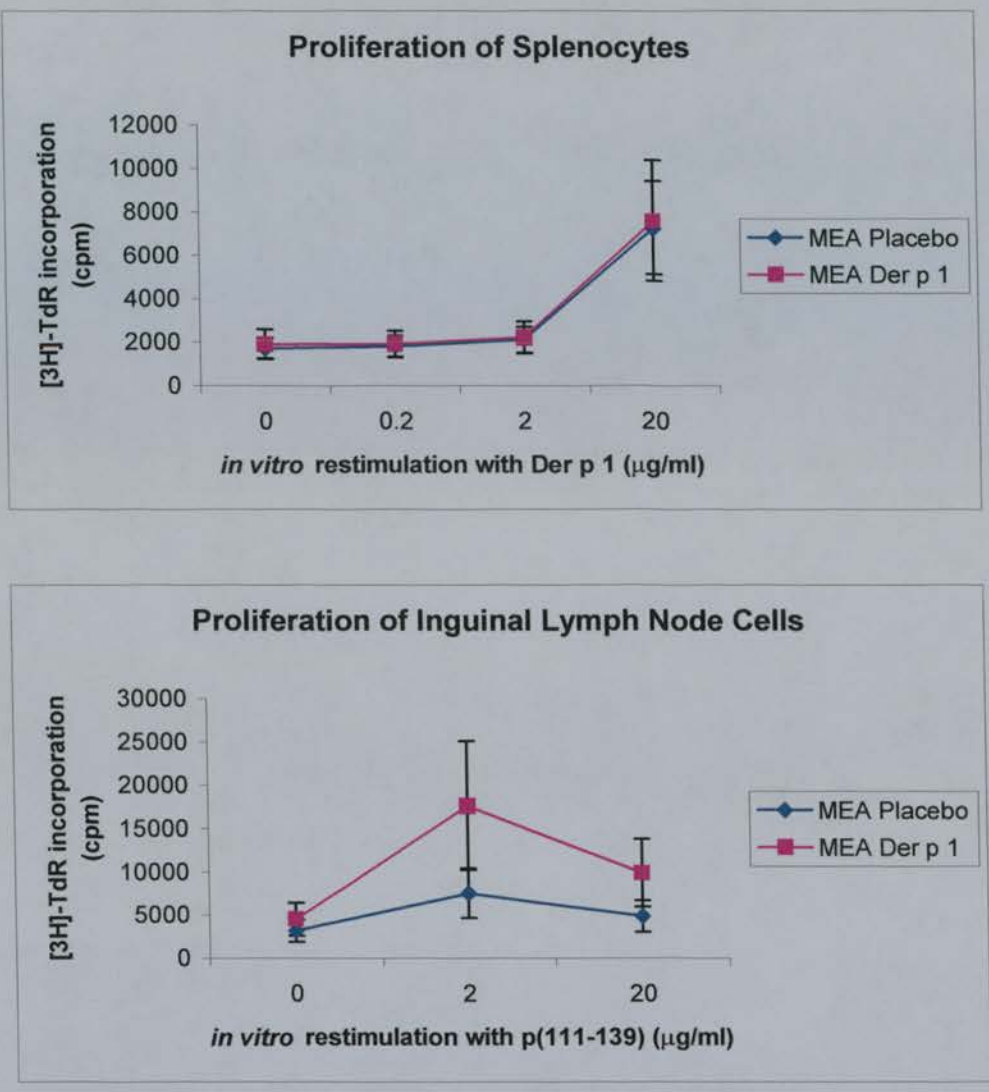
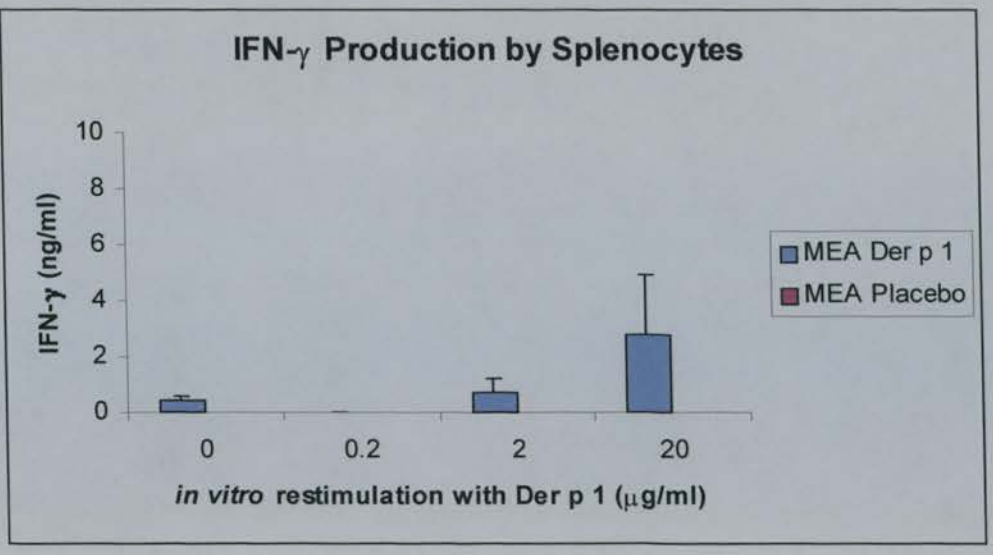


Figure 4.17. Assessment of tolerance induction after oral administration with MEA Der p 1.

Proliferation of splenocytes and inguinal lymph node cells.

C57BL/6 mice were given placebo or MEA Der p 1 by oral gavage on 3 consecutive days. 33 days after the last oral administration, the mice received a 50 µg dose of Der p 1 in CFA. The spleens and inguinal lymph nodes were removed 10 days after Der p 1/CFA administration. Single cell suspensions (4×10^5/well splenocytes and 2×10^5/well inguinal lymph node cells) were cultured with media alone or in the presence of 0.2, 2 or 20 µg/ml Der p 1. Proliferation was measured at 72 hours after pulsing with thymidine at 66 hours.

Mean +/- SE is shown.



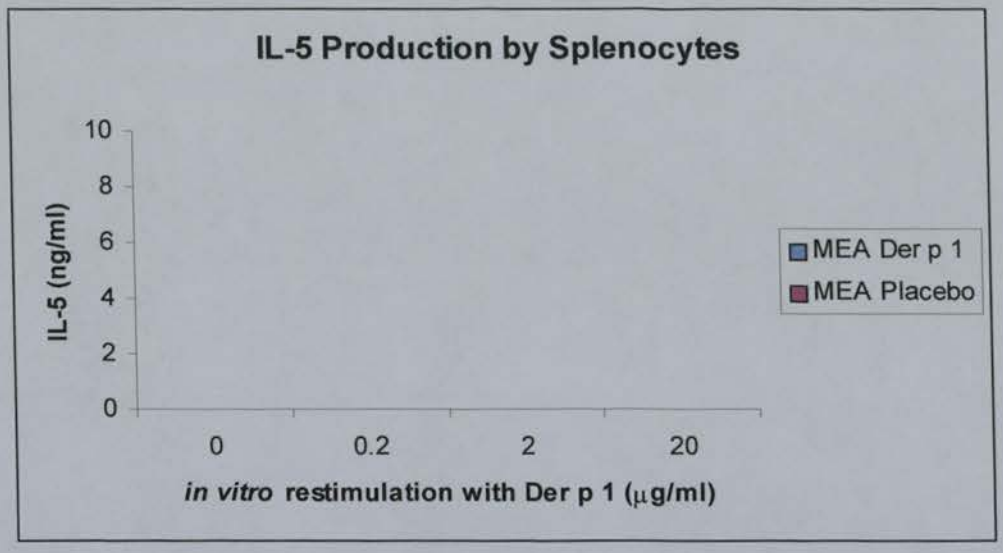


Figure 4.18. Assessment of tolerance induction after oral administration with MEA Der p 1.

IFN-γ and IL-5 production by splenocytes.

Splenocytes (4 x 10^5/well) were cultured in media alone or in the presence of 0.2, 2 or 20 μg/ml Der p 1. Supernatants were removed at 48 hours and frozen at -20°C until analysis by ELISA.

Mean +/- SE is shown.

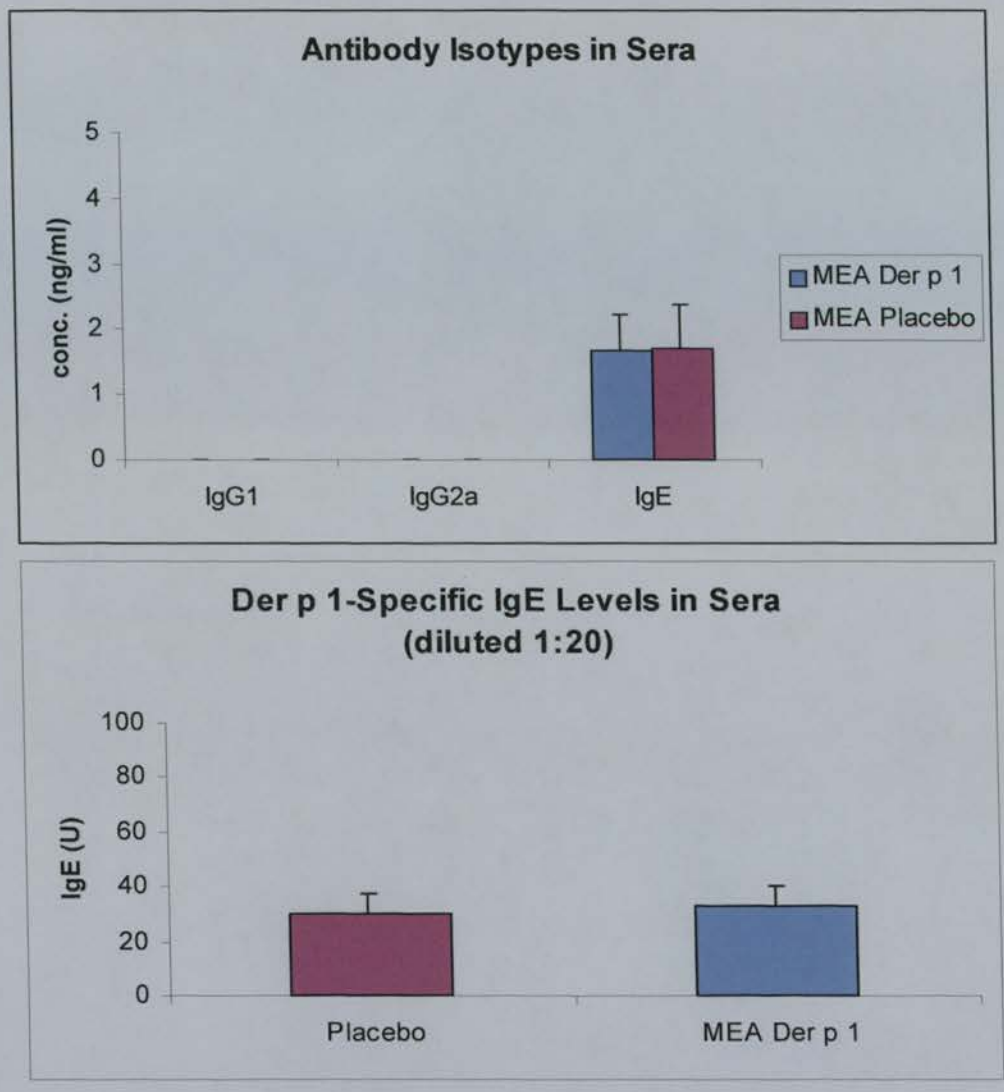


Figure 4.19. Assessment of tolerance induction after oral administration with MEA Der p 1.
Der p 1 – specific IgG1, IgG2a, IgE and total IgE levels in sera of mice.
The presence of antibodies was detected by ELISA.
The sera was diluted 1:1000 for upper chart and 1:20 for lower chart.
Mean +/- SE is shown.

4.8 Investigating whether the intranasal instillation of MEA Der p 1;p111-139 before sensitisation protect against the induction of Th2 mediated pulmonary inflammation on sensitisation and challenge with antigen

The intranasal route is an attractive route for the mucosal delivery of vaccines. The nasal epithelium is accessible, is less exclusive to high molecular molecules than in other mucosa, and its mucus covering contains considerably lower levels of proteolytic enzymes compared with the gut. Furthermore it is now proven that nasal associated lymphoid tissues (NALT) have a lymphoepithelium, which contain M-cells for selective antigen uptake [473]. Intranasal administration of encapsulated *Yersinia pestis* subunits protected mice from an aerosol challenge with a virulent strain of pneumonic plague [474]. The predominance of IgG1 in sera suggested that the response was Th2-skewed. An early study by Yan et al demonstrated high systemic and long-persisting IgG2a and local mucosal (sIgA) immunity against aerosol challenge with ricin toxoid vaccine encapsulated in polymeric microspheres after intranasal administration [475].

In this study, intranasal immunisation with microencapsulated Der p 1 peptide, p(111-139) was performed with the aim of protecting mice from subsequent allergic challenge with Der p 1 via the airways. There were four groups of mice in the study: saline alone (n =5), peptide p(111-139) alone (n = 5), microencapsulated peptide (MEA Der p 1;p111-139) (n = 7) and placebo microparticles (n = 4). The protocol is shown in detail on the page 157 but briefly, mice were given the appropriate intranasal treatment for five consecutive days. Mice were lightly anaesthetised with halothane gas and a micro-pipette was used to instil 30 µl liquid into the nostrils. At days 16 and 28 the mice received an i.p. immunisation of 10 µg Der p 1 in alum. At days 13 and 16 after the last i.p. immunisation, the airways of the mice were challenged with 50 µg Der p 1 by intratracheal immunisation. The mice were sacrificed 24 hours after the last i.t. challenge. Lung lavage was immediately performed *in situ* after death in order to obtain BAL fluid for cytospins and cytokine determination. Whole lungs were removed to determine the extent of eosinophil infiltration, and sera was taken to determine the presence of Der p 1-specific IgG1,

IgG2a and IgE antibodies. Tracheal lymph nodes were removed and pooled within groups along with individual spleens. Single cell suspensions were prepared from each mouse for *in vitro* restimulation with media alone or media containing 20 μg/ml Der p 1, Der p 1;p111-139, Der p 1;p111-119 ($CD8^+$ T cell epitope), Der p 1;p113-127 ($CD4^+$ epitope), OVA peptide (an unrelated antigen) and α-CD3/CD28 antibodies (as a positive control, 5 μg/ml:2.5 μg/ml respectively).

Cytokine production by allergen reactive T cells isolated from mice treated prophylacticaly by intranasal peptide administration.

Figure 4.20 shows IL-5 production by splenocytes after *in vitro* restimulation with Der p 1, Der p 1;p111-139 and Der p 1;p113-127. The other *in vitro* stimuli are not included in this figure (and in figs. 4.21-4.23) as cytokine production by these splenocytes was not higher than background levels. IL-5 was produced by splenocytes restimulated with α-CD3/CD28 antibodies (data are not shown). Mice treated with MEA peptide showed a 69% reduction in IL-5 levels as compared with the saline control group and a 62% reduction as compared to mice receiving peptide alone after restimulation *in vitro* with Der p 1. When splenocytes were restimulated *in vitro* with Der p 1;p111-139 and Der p 1;p113-127, mice receiving MEA peptide showed a reduction in IL-5 of 51% and 75% respectively compared to saline controls. The threshold of sensitivity for the IL-5 ELISA was around 50pg/ml.

Figure 4.21 shows IL-13 production by splenocytes after *in vitro* restimulation with Der p 1, Der p 1;p(111-139) and Der p 1;p(113-127). With the exception of the placebo group, most mice fall within a tight range. IL-13 production by MEA peptide mice was comparable to the saline controls. The threshold of sensitivity for the IL-13 ELISA was around 20 pg/ml.

Results observed for the placebo mice in figures 4.20 (IL-5) and 4.21 (IL-13) are quite intriguing. The level of IL-5 and IL-13 production suggested that a Th2 response was being induced. However, the experiment was only undertaken once and only four mice were tested. Also, the largest inter-group variation was observed in the placebo mice, therefore, it would be preferable to repeat the experiment before any firm conclusions can be drawn on the effect of placebo microparticles on Th2 induction.

Figure 4.22 shows IFN-γ production by splenocytes after *in vitro* restimulation with Der p 1, Der p 1;p111-139 and Der p 1;p113-127. Mice treated with MEA peptide showed an 82% reduction in IFN-γ compared with the saline control group, and a 75% reduction in IFN-γ was observed compared to the peptide treated group. The threshold of sensitivity for the IFN-γ ELISA was around 20 pg/ml.

Figure 4.23 shows IL-10 production by splenocytes after *in vitro* restimulation with Der p 1, Der p 1;p(111-139) and Der p 1;p(113-127). Production of IL-10 followed a similar trend to IL-5 and IL-13 production. Placebo mice showed the highest levels of IL-10, whereas MEA peptide mice showed the least but this was only marginally less than the saline controls. The threshold of sensitivity for the IL-10 ELISA was around 20 pg/ml.

IL-5 and IL-13 production by TLN cells is shown in figure 4.24. The highest levels of each cytokine are produced by the placebo-immunised mice. MEA peptide-immunised mice show a marked reduction in the production of these cytokines compared to the peptide-immunised mice (66% - IL-5, 55% - IL-13), however levels are still higher than the saline-immunised mice.

TLN cells from the saline- and peptide-immunised mice produce the highest amounts of IFN-γ (figure 4.25). This suggests that the decreased production of IL-5 observed in the MEA peptide-immunised mice compared with peptide alone, is not due to a Th2/Th0 → Th1/Th0 shift. IFN-γ production by splenocytes shows a similar pattern and thus supports this fact. There was an increase in levels of IL-10 produced by antigen specific T cells obtained from the draining lymph nodes of mice treated intranasally with MEA Der p 1;p111-139 or Der p 1;p111-139 peptide alone, as compared with cells obtained from saline controls. Comparisons of the ratio of IL-5 to IL-10, revealed a significant increase in the ratio of IL-10 (3.3:1 for MEA peptide mice compared to 7.7:1 for saline mice).

Again, there is evidence of a considerable placebo effect. As mentioned earlier, the experiment was only undertaken once and only four mice were contained within the placebo group. The lymph nodes were pooled before culturing with Der p 1, so the observed effect may be due to one or two of the mice producing large amounts of IL-10 thus impacting on the group as a whole.

Effect of intra-nasal peptide administration on eosinophil levels in Der p 1 allergen challenged airways.

Figure 4.26 shows the extent of eosinophil infiltration into the lungs of mice. Immediately, it is apparent that the number of eosinophils present in individual mice vary widely within each group. The eosinophil peroxidase assay is quite a crude method to measure the extent of eosinophilia and large inter-group variation can often be attributed to: lavage technique, weight of lungs (varies due to dryness of lungs) and loss of material to the homogenisation process. One mouse from the saline group was not included as removal of lavage fluid had been unsuccessful. We have found previously that this can lead to large differences when compared to successfully lavaged mice. Despite large variations within groups, a clear trend can be observed in that MEA peptide-immunised mice have a significant reduction in eosinophilia (54% compared to saline controls ($P < 0.01$) and 57% compared to peptide). This correlates well with TLN IL-5 and IL-13 production.

The presence of IL-5 and IL-4 was also determined from BAL fluid (figure 4.27). There was little difference between the groups for IL-4 production but IL-5 production by MEA peptide mice was reduced by 62% compared to saline controls, which supports the trend observed in eosinophilia levels.

The reduction in eosinophilia coupled with the trend in cytokine production was therefore due to a loss of antigen specific T cell responsiveness and eosinophil recruitment, rather than a Th1 shift in cytokine production. The loss of T cell responsiveness to intact Der p 1, following mucosal tolerance induction with an immunodominant T cell epitope (Der p 1;p111-139) , may be due to linked suppression resulting in a loss of responsiveness to minor T cell epitopes within Der p 1. Peptide alone failed to tolerise Der p 1 reactive T cells, suggesting that MEA particles enhanced the tolerogenicity of the peptide.

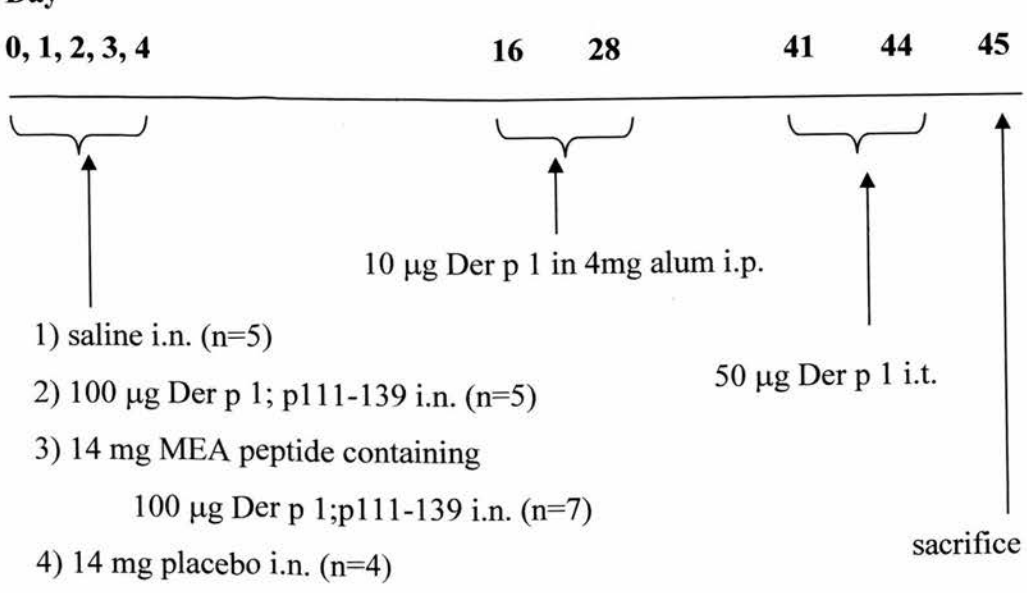
Effect of intranasal peptide treatment on antigen specific immunoglobulin levels induced following systemic immunisation.

To determine whether the i.n. administration of a single T cell epitope peptide could influence B cell responses, Der p 1 specific antibody levels in sera were measured by ELISA. Der p 1-specific IgG1, IgG2a and IgE levels in sera are shown

in figure 4.28. Der p 1-specific IgE, IgG1 and IgG2a levels were not reduced in mice receiving MEA peptide compared with saline controls, although there was a 74% reduction in Der p 1-specific IgG1 levels, in sera obtained from Der p 1;p111-139 treated mice. Small amounts of IgG2a (100-fold less than IgG1) were detected in all mice but there were no significant differences between groups. Der p 1-specific IgE was also determined using standard sera from mice immunised systemically with Der p 1 in alum, to assign arbitrary values to the levels of IgE observed. The placebo-immunised mice produced the greatest amount of IgE, although variation within this group was quite large. This may be linked to the non-specific reduction in IFN-γ production. There was no significant difference between the other groups of mice.

Investigating whether the intranasal instillation of MEA Der p 1;p111-139 before sensitisation protects against the induction of Th2 mediated pulmonary inflammation on sensitisation and challenge with antigen.

Protocol



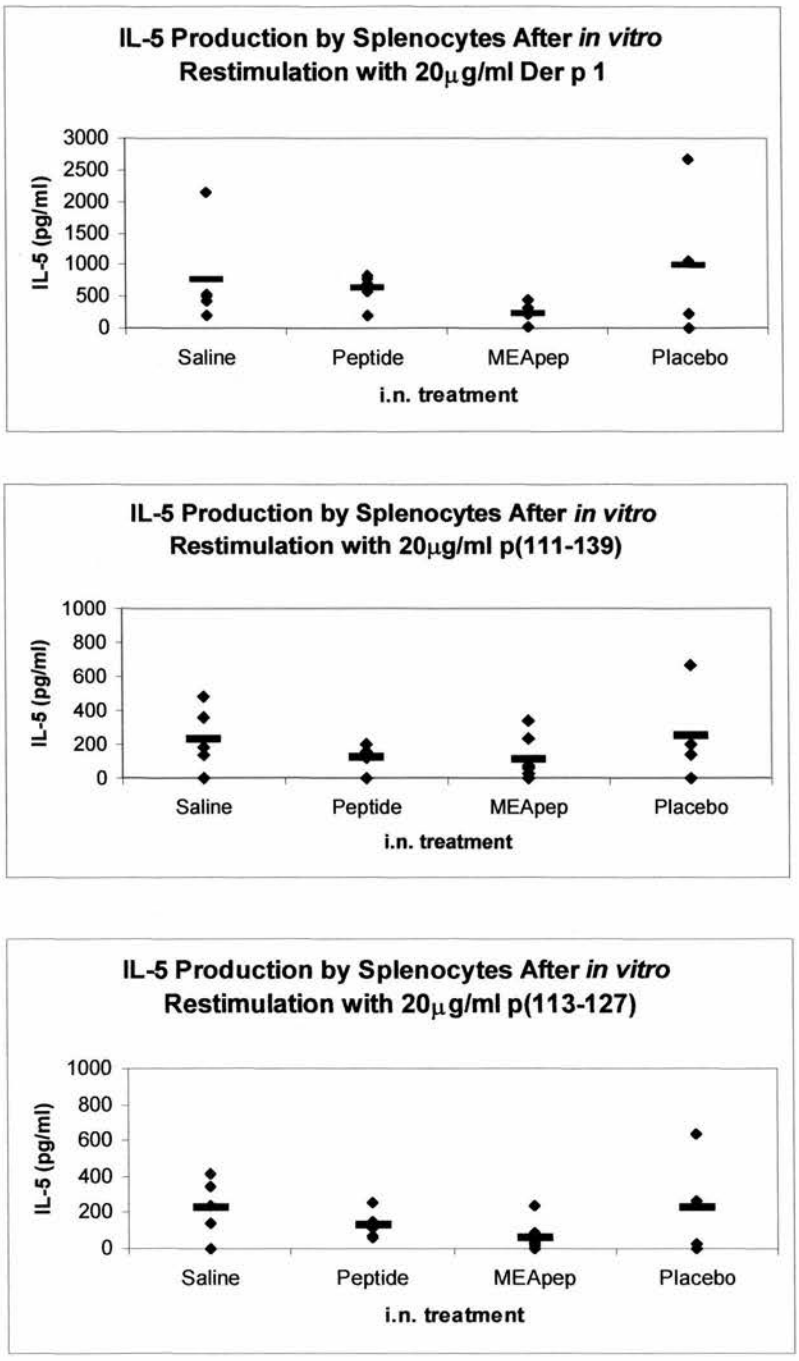


Figure. 4.20. IL-5 production by splenocytes.
C57BL/6 mice were given saline alone, Der p 1;p111-139 peptide alone, MEA Der p 1;p111-139 or placebo microparticles by intranasal instillation. Single cell suspensions (5 x 10^5/well splenocytes) were cultured with media alone or in the presence of 20 μg/ml Der p 1, Der p 1;p111-139, Der p 1;p111-119, Der p 1;p113-127, OVA peptide and α-CD3/CD28 antibodies. Supernatants were removed at 72 hours for cytokine determination. The graphs represent individual mice (diamonds) as well as mean values within each experimental group (lines).

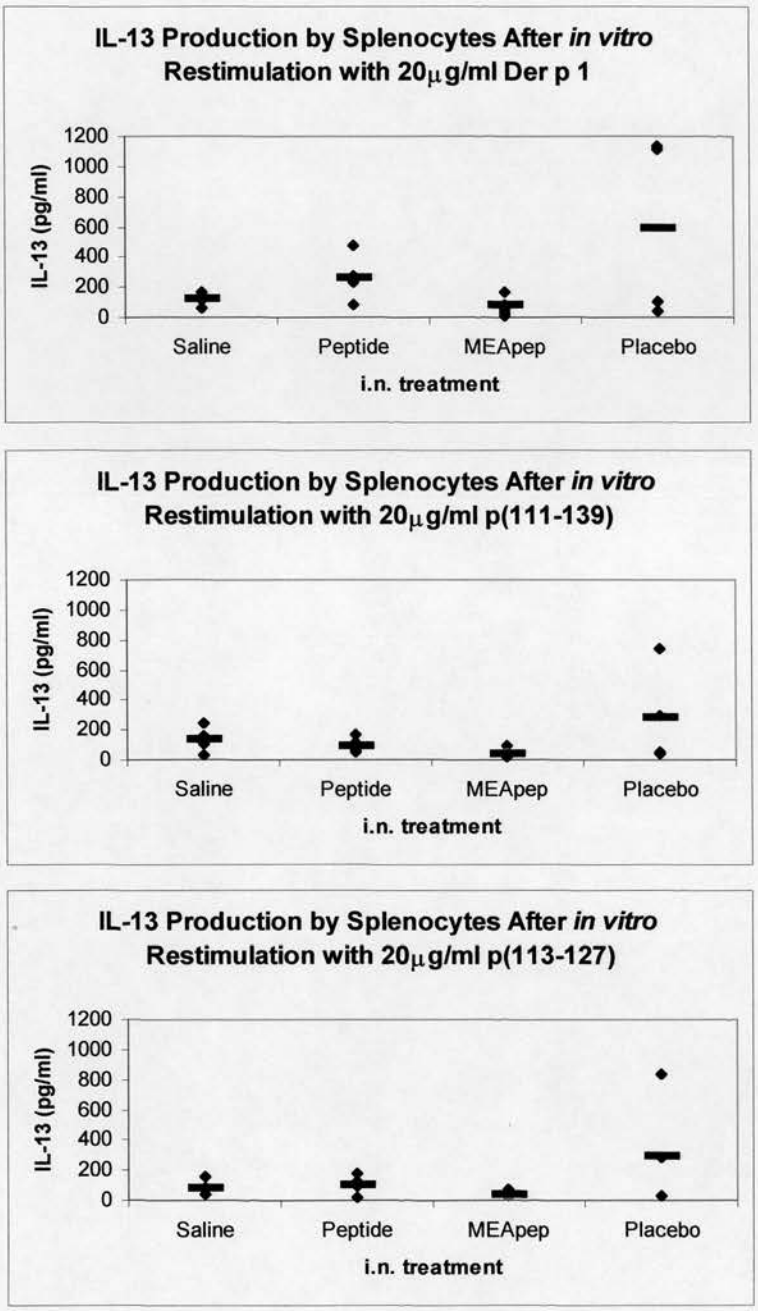


Figure 4.21. IL-13 production by splenocytes.
C57BL/6 mice were given saline alone, Der p 1;p111-139 alone, MEA Der p 1;p111-139 or placebo microparticles by intranasal instillation. Single cell suspensions (5 x 10^5/well splenocytes) were cultured with media alone or media supplemented with 20 μg/ml Der p 1, Der p 1;p111-139, Der p 1;p111-119, Der p 1;p113-127, OVA peptide and α-CD3/CD28 antibodies. Supernatants were removed at 72 hours for cytokine determination. The graphs represent individual mice (diamonds) as well as mean values within each experimental group (lines).

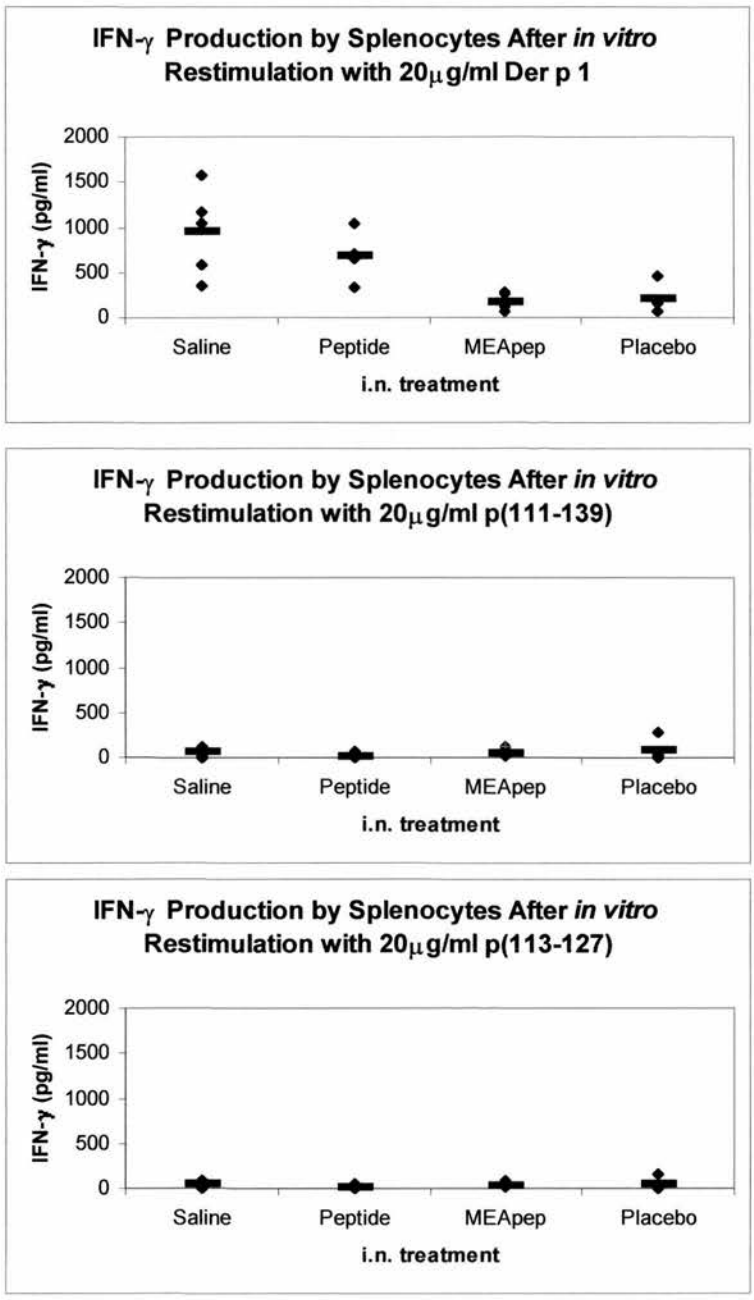


Figure 4.22. IFN-γ production by splenocytes.
C57BL/6 mice were given saline alone, Der p 1;p111-139 alone, MEA Der p 1;p111-139 or placebo microparticles by intranasal instillation. Single cell suspensions (5 x 10^5/well splenocytes) were cultured with media alone or in the presence of 20 μg/ml Der p 1, Der p 1;p111-139, Der p 1;p111-119, Der p 1;p113-127, OVA peptide and α-CD3/CD28 antibodies. Supernatants were removed at 72 hours for cytokine determination. The graphs represent individual mice (diamonds) as well as mean values within each experimental group (lines).

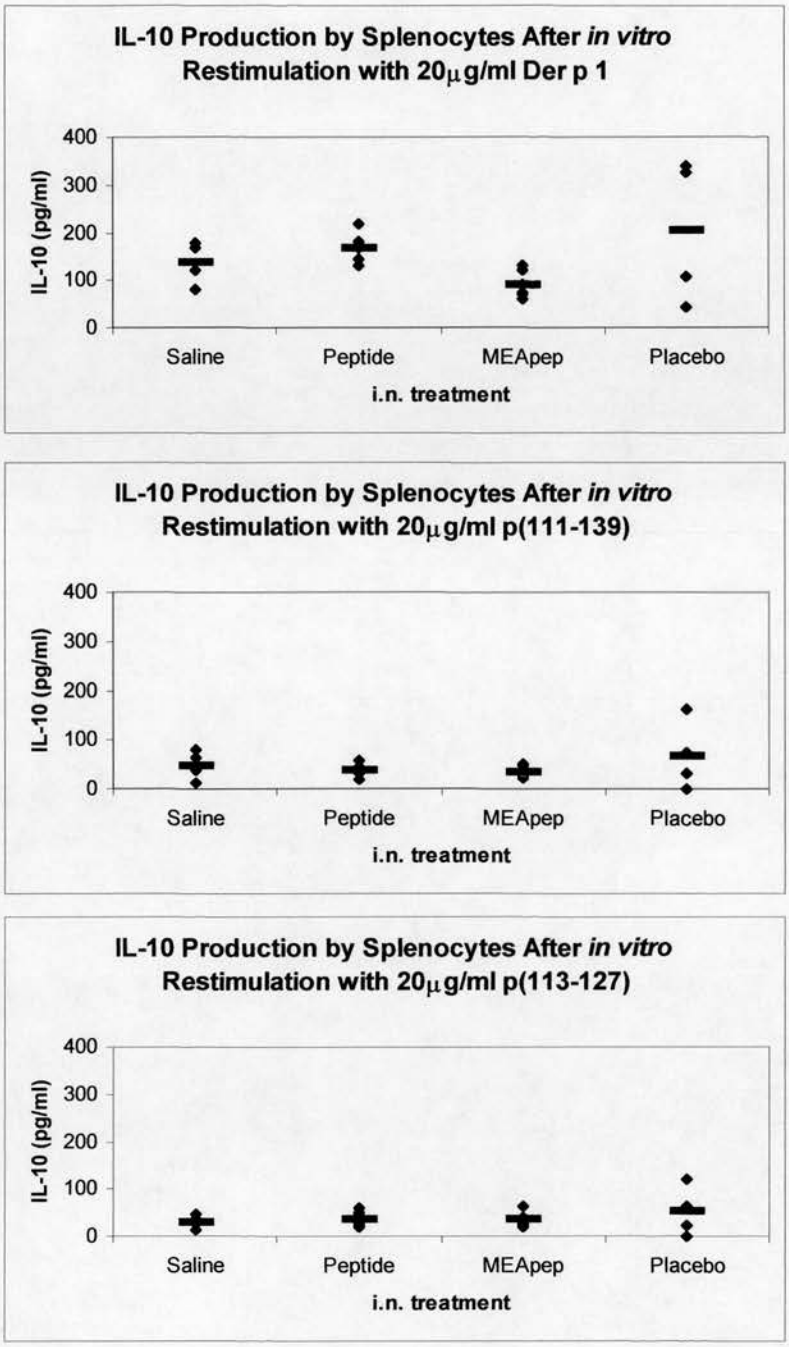


Figure 4.23. IL-10 production by splenocytes.
C57BL/6 mice were given saline alone, Der p 1;p111-139 alone, MEA Der p 1;p111-139 or placebo microparticles by intranasal instillation. Single cell suspensions (5 x 10^5/well splenocytes) were cultured with media alone or in the presence of 20 μg/ml Der p 1, Der p 1;p111-139, Der p 1;p111-119, Der p 1;p113-127, OVA peptide and α-CD3/CD28 antibodies. Supernatants were removed at 72 hours for cytokine determination. The graphs represent individual mice (diamonds) as well as mean values within each experimental group (lines).

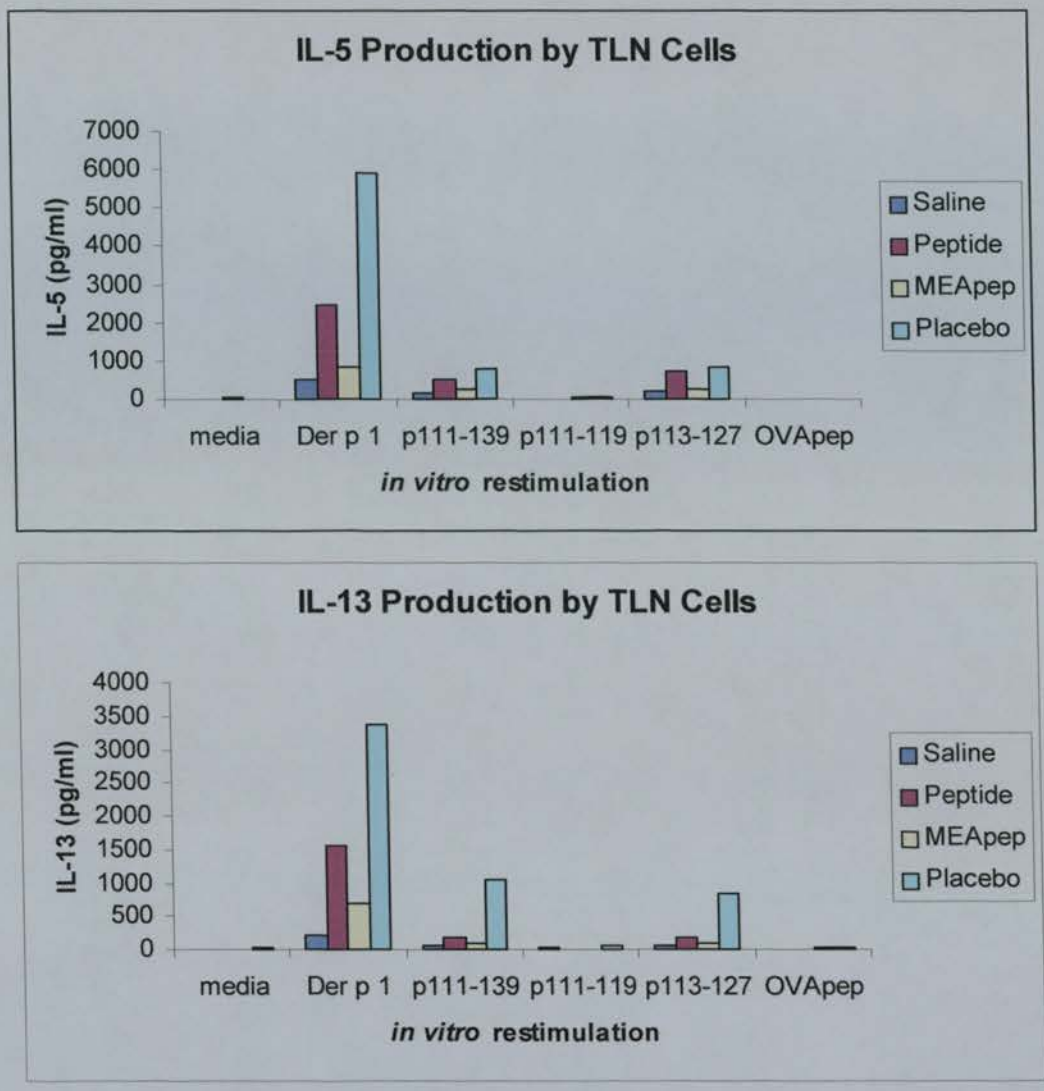
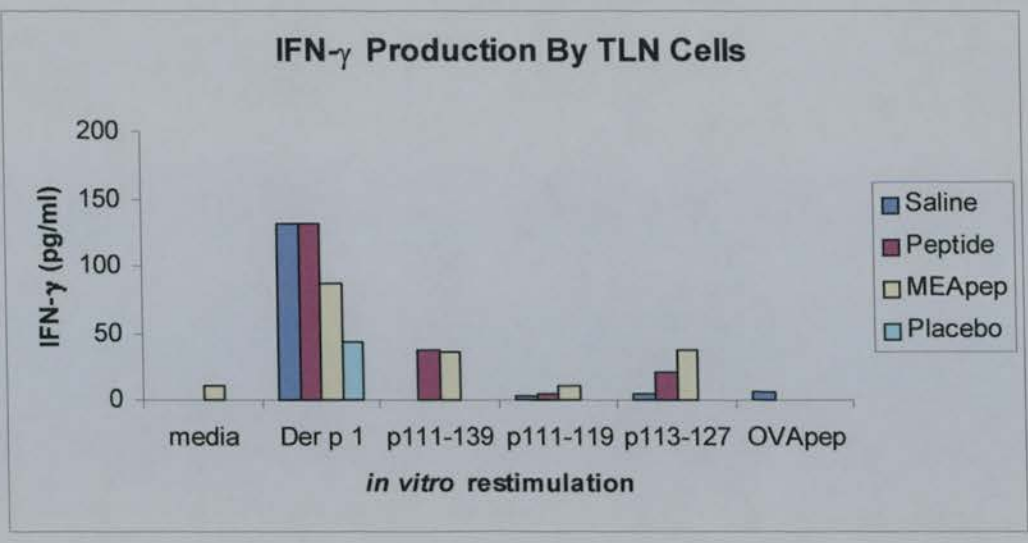


Figure 4.24. Investigating whether the intranasal instillation of MEA Der p 1;p111-139 before sensitisation protects against the induction of Th2 mediated pulmonary inflammation on sensitisation and challenge with antigen. IL-5 and IL-13 production by TLN cells.

C57BL/6 mice were given saline alone, Der p 1;p111-139 alone, MEA Der p 1; p111-139 or placebo microparticles by intranasal instillation. Single cell suspensions from pooled tracheal lymph nodes (5 x 10^5/well TLN cells) were cultured with media alone or in the presence of 20 μg/ml Der p 1, Der p 1;p111-139, Der p 1;p111-119, Der p 1;p113-127, OVA peptide and α-CD3/CD28 antibodies. Supernatants were removed at 72 hours for cytokine determination. Each sample is shown in duplicate.



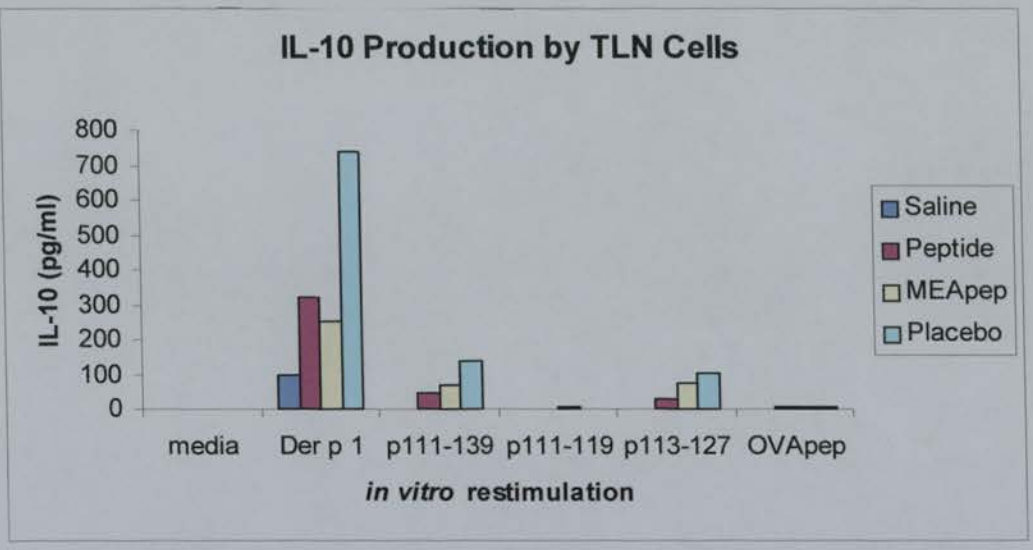


Figure 4.25. Investigating whether the intranasal instillation of MEA Der p 1;p111-139 before sensitisation protects against the induction of Th2 mediated pulmonary inflammation on sensitisation and challenge with antigen. IFN-γ and IL-10 production by TLN cells.

C57BL/6 mice were given saline alone, Der p 1;p111-139 alone, MEA Der p 1;p111-139 or placebo microparticles by intranasal instillation. Single cell suspensions from pooled tracheal lymph nodes (5 x 10^5/well TLN cells) were cultured with media alone or in the presence of 20 μg/ml Der p 1, Der p 1;p111-139, Der p 1;p111-119, Der p 1;p113-127, OVA peptide and α-CD3/CD28 antibodies. Supernatants were removed at 72 hours for cytokine determination.

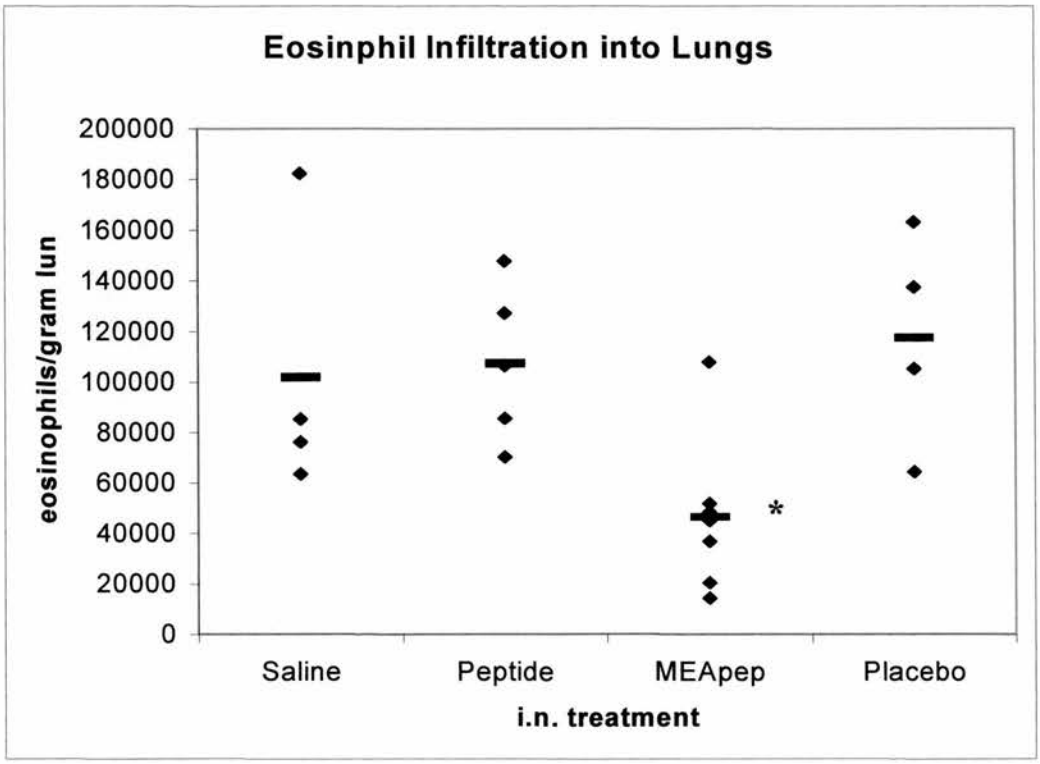


Figure 4.26. Investigating whether the intranasal instillation of MEA Der p 1;p111-139 before sensitisation protects against the induction of Th2 mediated pulmonary inflammation on sensitisation and challenge with antigen.
Eosinophil Infiltration into Lungs
Eosinophils were detected by an eosinophil peroxidase assay.
Individual mice are represented by diamonds, mean values within each group are represented by bars. Differences in means were significant (* $P < 0.01$).

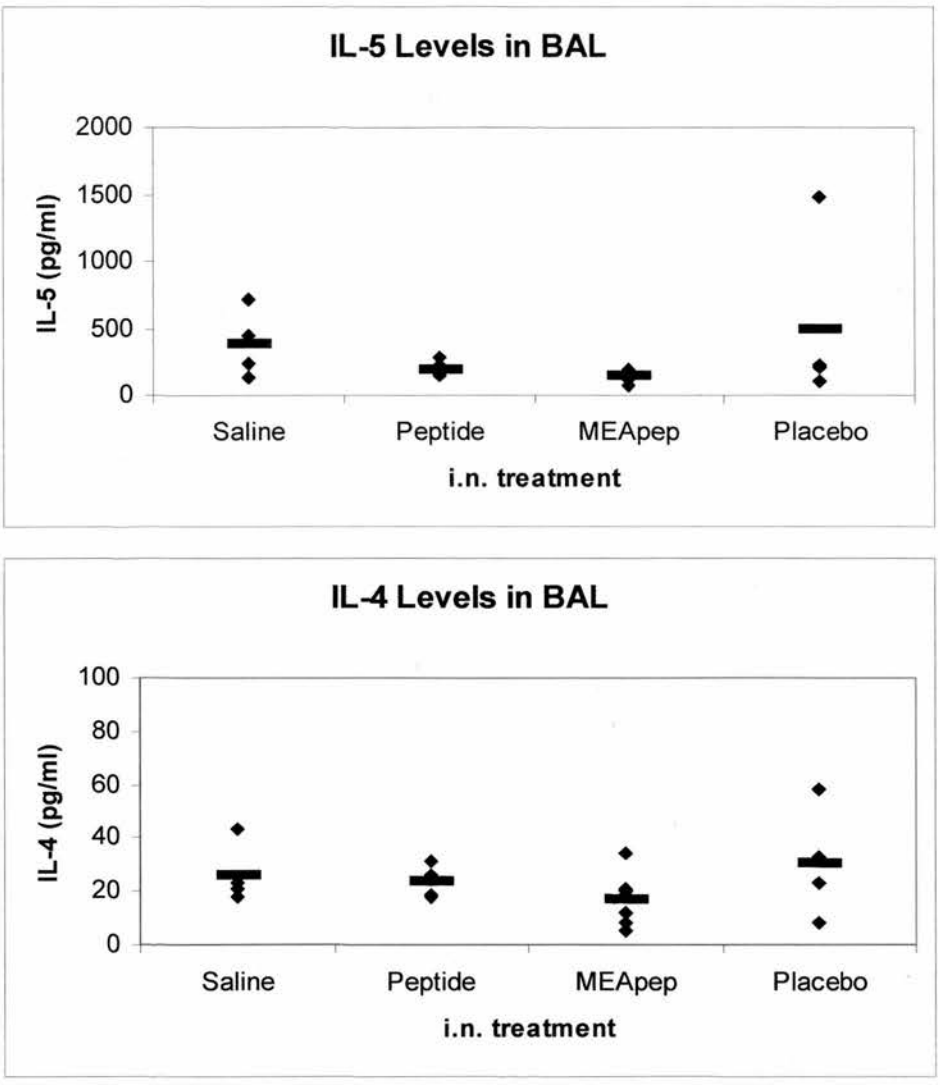


Figure 4.27. Investigating whether the intranasal instillation of MEA Der p 1;p111-139 before sensitisation protects against the induction of Th2 mediated pulmonary inflammation on sensitisation and challenge with antigen. IL-5 and IL-4 levels in BAL fluid.

Mouse lungs were lavaged in situ after death with PBS. The lavage was centrifuged to remove the cellular infiltrate (used for cytospins). The supernatant was frozen at -70° until analysis by ELISA. The charts represent individual mice (diamonds) as well as mean values within each experimental group (lines).

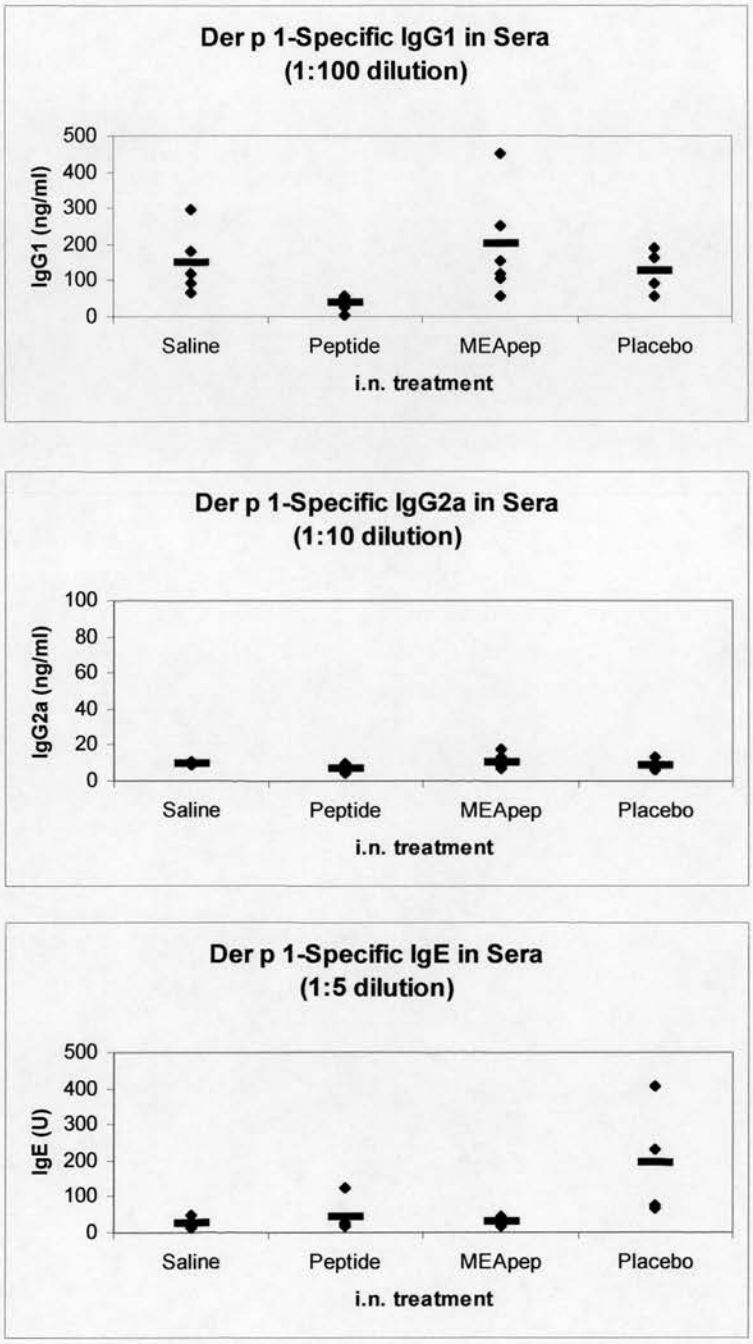


Figure 4.28. Investigating whether the intranasal instillation of MEA Der p 1;p111-139 before sensitisation protects against the induction of Th2 mediated pulmonary inflammation on sensitisation and challenge with antigen?
Der p 1-Specific IgG1, IgG2a and IgE levels in sera.
The graphs represent individual mice (diamonds) as well as mean values within each experimental group (lines).

4.9 Investigating whether the intranasal instillation of MEA Der p 1 prevents the induction of airway inflammatory responses in mice sensitised by systemic immunisation with Der p 1.

The purpose of the experiments described here was to investigate whether the intranasal administration of MEA Der p 1 microparticles could down-regulate established Th2 cytokine mediated allergic responses.

The immunisation protocol was chosen because we wanted to investigate a therapeutic (treatment) regimen which involves sensitising mice to Der p 1 using an adjuvant free protocol over the prophylactic regimen where mice are immunised with Der p 1 in alum. The protocol is shown in detail on page 169. Mice were immunised systemically by i.p. injection of 10 μg concentrations of Der p 1 in saline. Sixteen days after the last immunisation, groups of mice received either saline alone (Group A, n = 4), placebo particles (Group B, n = 4), Der p 1 alone (Group C, n = 4) or MEA Der p 1 (Group D, n = 4) particles by intranasal instillation on 5 consecutive days. After a further seven days, the mice were challenged by intra-tracheal instillation of Der p 1 allergen into the airways (see protocol) and were then sacrificed 24 hours after the final intra-tracheal challenge.

Figure 4.29 shows the splenocyte proliferative recall responses to a number of *in vitro* stimuli. Single cell suspensions were restimulated with either Der p 1, defined immunodominant epitopes of Der p 1 - p(111-139) (this peptide contains both the CD4 and CD8 T cell epitopes), p(111-119) (the CD8 epitope), p(113-127) (the CD4 epitope) or with ovalbumin (an irrelevant protein). All protein and peptide concentrations were used at 10μg/ml. Each of the four groups of mice showed a small degree of priming in response to Der p 1, with the strongest proliferative responses exhibited by the mice immunised with MEA Der p 1 (S.I. 2.8) and Der p 1 (S.I. 3.4). The degree of priming in the saline- and placebo-immunised groups were similar (S.I. 1.9, 1.4 respectively). In all groups of mice response to Der p 1 was the strongest of the different stimuli. Proliferative responses to the three peptides were

similar in magnitude, with no statistically significant differences between the four groups (S.I. in the range 1 – 2).

Proliferation in tracheal lymph node cell cultures was certainly more marked than splenocyte cultures (figures 4.30-4.31). The highest levels by far were exhibited by the Der p 1 group of mice (note scale). Restimulation with Der p 1 resulted in memory responses five times higher than the saline group. Again it was difficult to differentiate between which epitope (CD4 or CD8) induced the greatest response in all groups of mice.

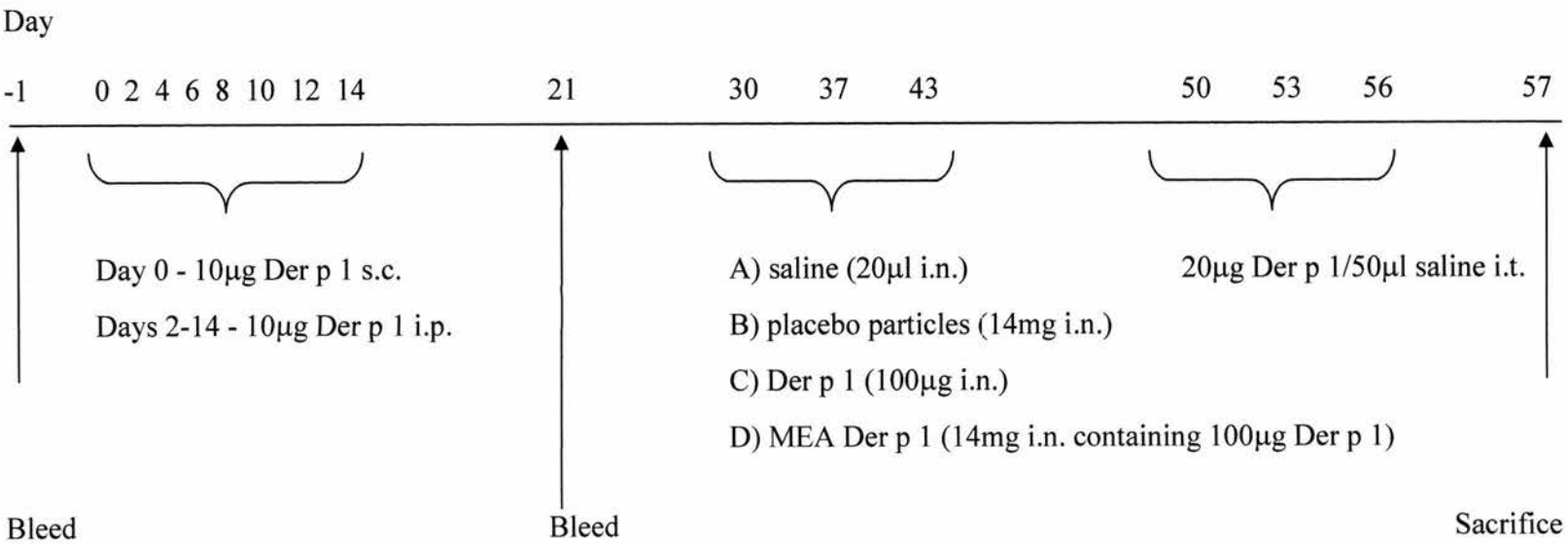
IFN-γ and IL-5 levels were measured in the culture supernatants which were removed at 72 hours. Neither cytokine was detectable in either splenocyte or lymph node cultures which was strange as we would have expected to see some existence of cytokines.

Marked levels of Der p 1-specific IgG1 were detected in all groups of mice but were highest in the Der p 1 and MEA Der p 1 groups (figure 4.32). Der p 1-specific IgG2a was also detected in all groups but this was considerably reduced when compared to IgG1 levels (four-fold less for saline and placebo groups, ten-fold less for Der p 1 and MEA Der p 1). Der p 1-specific IgE was also tested but due to diminishing quantities of sera it was not possible to test every mouse; saline (n = 2), placebo (n = 2), Der p 1 (n = 4) and MEA Der p 1 (n = 3). IgE levels were highest in the placebo group but with a mean of only two mice this may not reflect an accurate result. There was little difference between Der p 1 and MEA Der p 1 but IgE levels in both groups were considerably elevated compared to saline controls.

The highest levels of lung eosinophilia were detected in the MEA Der p 1 and the Der p 1 groups of mice (figure 4.33). These levels were approximately 3 times higher than those detected in the saline and placebo groups.

Investigating whether the intra-nasal instillation of MEA Der p 1 prevents the induction of airway inflammatory responses in mice sensitised by systemic immunisation with Der p 1.

Protocol



Group A - n=4

Group B – n=4

Group C – n=5

Group D – n=5

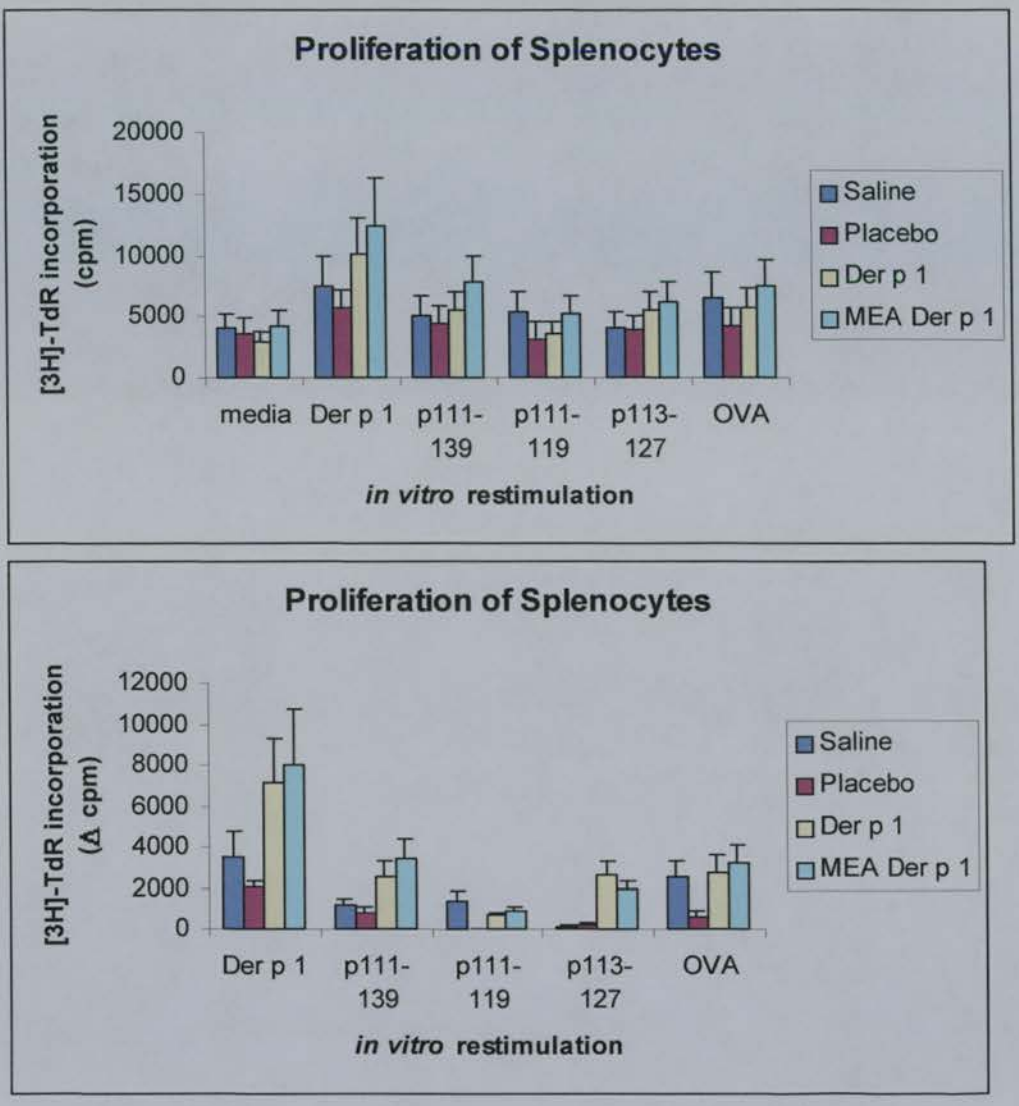


Figure 4.29. Investigating whether the intranasal instillation of MEA Der p 1 prevents the induction of airway inflammatory responses in mice sensitised by systemic immunisation with Der p 1.

Proliferation of splenocytes (5 x 10^5 cells/well) after in-vitro stimulation with media alone or media supplemented with 10 μg/ml Der p 1, p(111-139), p(111-119), p(113-127) and OVA. Cells were cultured for 72 hours with the antigenic stimuli, pulsed with thymidine for 18 hours and then harvested.

Mean +/- SE is shown.

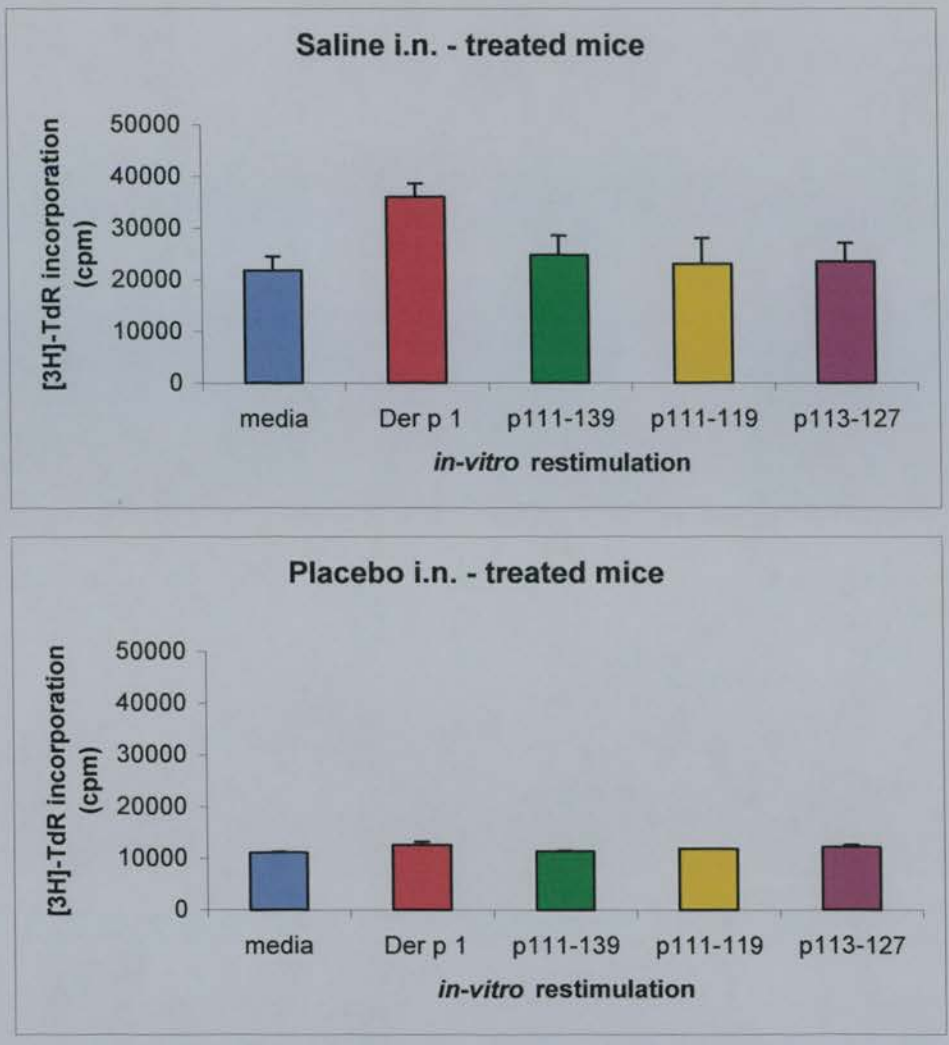


Figure 4.30. Investigating whether the intranasal instillation of MEA Der p 1 prevents the induction of airway inflammatory responses in mice sensitised by systemic immunisation with Der p 1.
Proliferation of lymphocytes (4 x 10^5 cells/well) after in-vitro stimulation with media alone or media supplemented with 10 μg/ml Der p 1, p(111-139), p(111-119) or p(113-127). Cells were cultured for 72 hours with the antigenic stimulus, pulsed with thymidine for 18 hours and then harvested.
Mean +/- SE is shown.

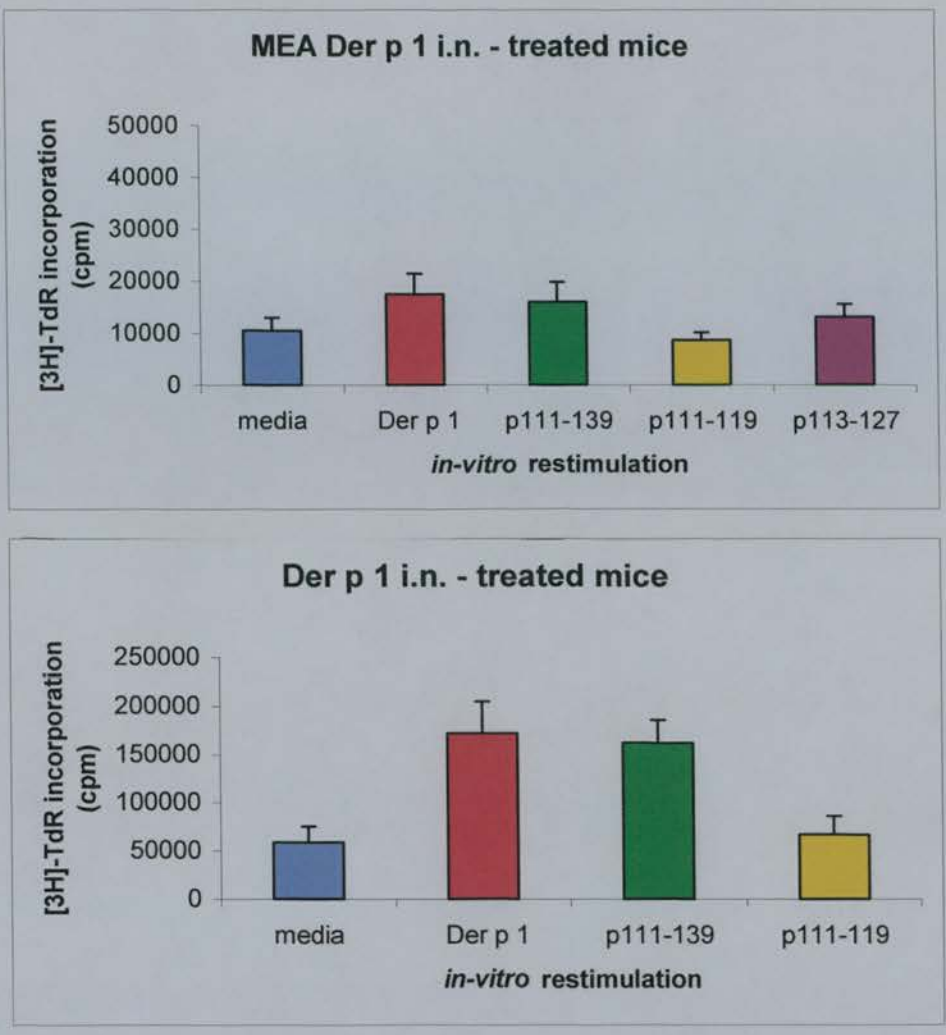


Figure 4.31. Investigating whether the intranasal instillation of MEA Der p 1 prevents the induction of airway inflammatory responses in mice sensitised by systemic immunisation with Der p 1.
Proliferation of lymphocytes (4 x 10^5 cells/well) after in-vitro stimulation with media alone or in the presence of 10 μg/ml Der p 1, Der p 1;p111-139, Der p 1;p111-119 or Der p 1;p113-127. Cells were cultured for 72 hours with the antigenic stimulus, pulsed with thymidine for 18 hours and then harvested.
(Note: there were insufficient cells from the Der p 1 group of mice to restimulate with Der p 1;p113-127.)
Mean +/- SE is shown.

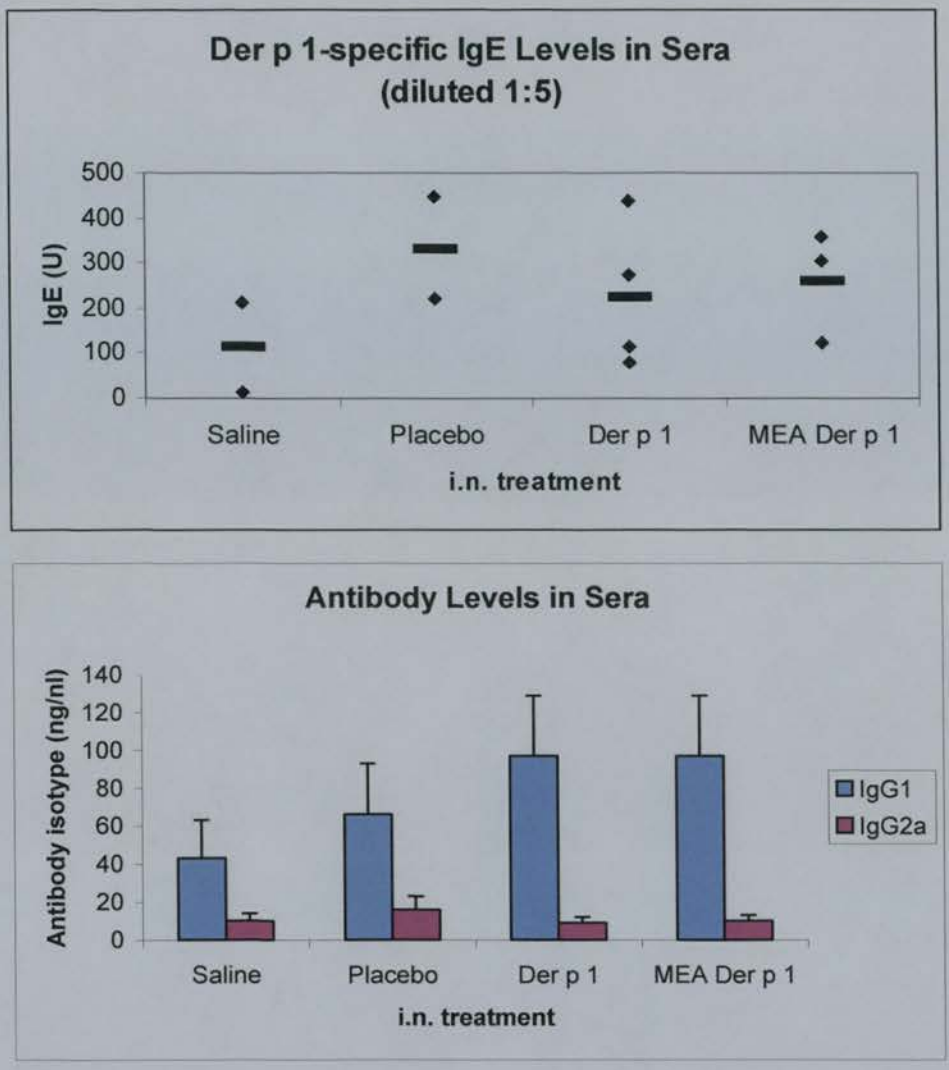


Figure 4.32. Investigating whether the intranasal instillation of MEA Der p 1 prevents the induction of airway inflammatory responses in mice sensitised by systemic immunisation with Der p 1.
Der p 1-specific IgG1, IgG2a and IgE levels in sera.
Antibody levels were detected by ELISA.
Sera was diluted 1:1000 for IgG1 and IgG2a. Sera was diluted 1:5 for IgE.
Mean +/- SE is shown.

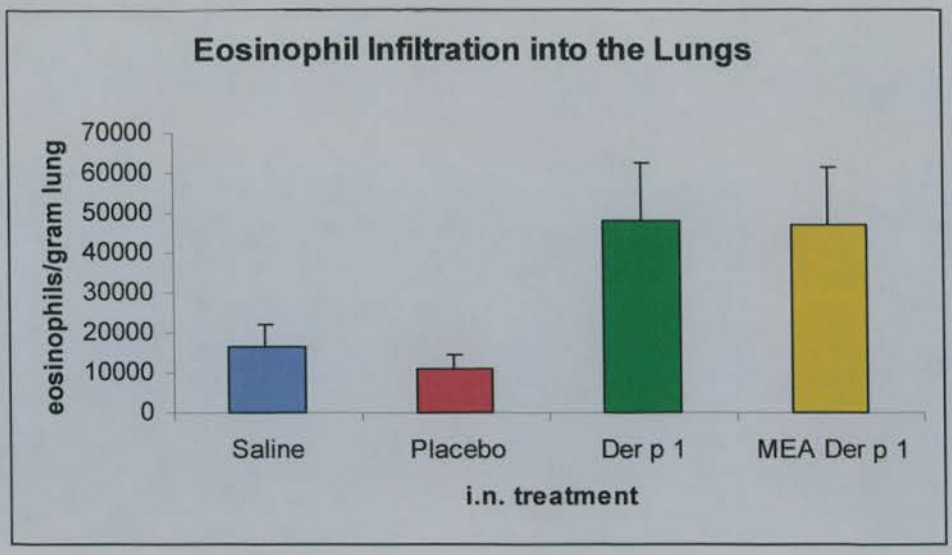


Figure 4.33. Investigating whether the intranasal instillation of MEA Der p 1 prevents the induction of airway inflammatory responses in mice sensitised by systemic immunisation with Der p 1.
Eosinophil Infiltration into Lungs
Eosinophils were detected by an eosinophil peroxidase assay.
Mean +/- SE is shown.

4.10 Discussion

The purpose of this study was to investigate the ability of MEA Der p 1 microparticles to induce an immune response, with the hope that these particles could eventually be utilised as a potential therapy for the alleviation of allergic disease, either by tolerance induction or immune deviation. These responses were generated *in vivo* by a number of immunisation protocols and then measured using established *in vitro* protocols. This involved restimulation with antigen after which proliferative responses and cytokine production were determined. The B cell response was also measured by determining the levels of antigen-specific antibody isotypes present in sera. Initially a number of experiments were undertaken in order to determine the most effective route of immunisation (sub-cutaneous, intra-peritoneal, intravenous, oral or intra-nasal). The routes of immunisation (i.p. and s.c.) that induced the greatest immune response were investigated further to determine optimum dosage and kinetics but these routes of administration were not used for subsequent experiments. The possibility of tolerance induction after oral administration of MEA particles was investigated, along with the effect of MEA Der p 1 on an established allergic response in a mouse model.

PLG microparticles are very stable if kept at 4°C in a moisture-deficient environment. After injection, microparticles induce only a minimal inflammatory response and degrade by non-enzymic hydrolysis to the normal metabolites, lactic and glycolic acids [453]. One mechanism of immune stimulation by PLG microparticles is thought to operate via a depot effect resulting from slow antigen release at the site of delivery. A second mechanism involves a more vigorous stimulation resulting from efficient intracellular delivery of high concentrations of antigen to APCs and subsequent presentation to T cells [462].

A study by O'Hagan *et al* demonstrated that the entrapment of the soluble protein OVA, normally a poor immunogen, in PLG microparticles resulted in the induction of potent serum IgG following i.p. or s.c. immunisation [476]. These IgG responses were significantly greater than or comparable to those induced with OVA and Freunds' adjuvant. The data in this study was in agreement with these findings, with high levels of antigen-specific IgG1 being detected 21 and 42 days after a single

s.c. or i.p. immunisation (figure 4.7) which was comparable to the response induced with Der p 1 in Freunds' adjuvant.

The induction of $CD4^+$ T-cell responses with MEA Der p 1 was demonstrated by proliferation and cytokine production by immune splenocytes restimulated *in vitro* with Der p 1. Immunisation with MEA Der p 1 by the i.p. route induced $CD4^+$ T cells that secreted high levels of IFN-γ in response to specific antigen stimulation *in vitro* (figure 4.12). This was in agreement with the findings of Moore *et al* who immunised mice with recombinant HIV protein entrapped in PLG microparticles [477]. The IFN-γ response was most predominant at day 9 of our study. By day 21 levels of IFN-γ were diminished and were virtually abolished by day 42 (figure 4.6). This was supported by the antigen-specific IgG1/IgG2a data with levels of IgG1 (an indicator of the presence of Th2 cytokines) increasing steadily as the time increased (figure 4.7). Unfortunately we were not able to support this further with IL-5 data. IL-5 production in the splenocyte culture supernatants was undetectable following each route (i.p. s.c. i.v.) of immunisation. From other unrelated experiments, I have found that proliferation levels must be significantly higher than those exhibited in this experiment for IL-5 to be detected. Therefore, it is suggested that IL-5 was produced by this population of $CD4^+$ T cells, especially at the later time-points but was beyond the threshold of detection by ELISA used in this study. This observation seemed to be supported by other PLG studies when only small amounts of IL-5 were detected after s.c. and i.p. immunisation with microparticles entrapping HIV proteins, however, the use of viral proteins in these studies would preferentially induce $CD8^+$ IFN-γ responses [477, 478]. Therefore, it is proposed that Th0/Th1 cells were induced at the early time-points after immunisation but a mixed Th1/Th2 population of $CD4^+$ T cells was induced at later time-points with Th2 $CD4^+$ T cells becoming more predominant by day 42.

Intra-peritoneal immunisation is thought to favour Th1 induction because this site is rich in macrophages which have been shown to act as APCs for Th1 cells [479]. It seems likely that phagocytosis by macrophages probably accounts for the ability of PLG microparticles to function as a potent antigen delivery systems, since macrophages process and present antigens together with class II molecules to T-helper cells to induce T-cell dependent immune responses [480]. Sub-cutaneous

administration of MEA particles delivers them to the extensive network of Langerhans cells that are situated under the skin. The Langerhans cells capture the antigen and then migrate to the draining lymph nodes where they mature and present the antigen to T helper cells in association with MHC class II. Therefore, phagocytosis of MEA Der p 1 particles probably resulted in the delivery of relatively large amounts of undegraded antigen intracellularly to APCs resulting in more effective antigen presentation.

A recent study by Ricciardi-Castagnoli and colleagues suggests an alternative mechanism for bacterial uptake in the gut mucosa other than by M cells [481]. They demonstrated that DCs could open the tight junctions between epithelial cells, send their dendrites outside the epithelium and directly sample bacteria. Although not at all proven, it may be possible that DCs could sample MEA Der p 1 microparticles in a similar manner. However, this mechanism is dependent on DCs expressing tight junction proteins, whose expression is regulated during the maturation process, which in turn, is induced by bacteria or bacterial LPS. For MEA particles to be taken up in this manner would suggest that the particles induced inflammation which is unlikely considering the dearth of evidence regarding there safe use *in vivo*.

After establishing the basic kinetics and identifying that the intra-peritoneal and sub-cutaneous routes induced the greatest immune response, further investigation concentrating on these routes was undertaken. These studies specifically concentrated on the dose of MEA Der p 1 with comparisons being made to the induction of immune responses with Der p 1 in CFA as a positive control.

Altering the dose of MEA Der p 1 within the range of 7-30 mg, did not have a marked effect on the immune response. However, good proliferative responses (2-3 times higher c.f. media control) were induced in all groups which were stronger than that induced with Der p 1 in CFA. When immunising subcutaneously, 14 mg MEA Der p 1 (containing approximately 100 μg Der p 1) induced the greatest level of IFN-γ production in the splenocyte cultures (figure 4.9). The same dose of MEA Der p 1 also induced IFN-γ production by splenocytes from intra-peritoneal immunised mice (figure 4.12). However, these levels were markedly reduced compared to sub-cutaneous immunisation. This may be attributable to the route of administration and also kinetics. The Langerhans cells in skin could prime T cell responses more

efficiently than DCs or macrophages in the peritoneal cavity. The subcutaneously-injected mice were sacrificed 11 days after immunisation whereas the intra-peritoneal mice were killed 21 days after immunisation. However, these finding do support the results from the previous experiment. IgG1 levels were highest in s.c. mice that had received 30 mg MEA Der p 1 (figure 4.10) and were evident in the 30 mg i.p. group only (figure 4.13). This could be explained by the ratio of the PLG polymer which was 60:40 lactide:glycolide. This ratio degrades relatively rapidly and releases the entrapped antigen over a few weeks. As more antigen became available for presentation and processing, in turn the memory B cell population became expanded and secreted more antigen-specific IgG1 antibodies. A large amount of evidence suggests that antibody class switching is induced by B cell activators in the presence of specific cytokines. IL-4, TGF-β and IFN-γ regulate isotype specificity of switching by regulating transcription of unrearranged or germline Ig C_H genes before class switching [482, 483]. IL-4 induces germline γ_1 and ε transcripts in mouse B cells and subsequent switching to these same isotypes [266, 484, 485]. Addition of IFN-γ to LPS-activated B cells induces germline γ_{2a} transcripts and subsequent switching to IgG2a in mouse B cells [486, 487]. In our system the predominance of IgG1 over IgG2a suggests that IL-4 would have been present in our cultures even though we failed to detect any. However, IL-4 is notoriously difficult to detect. Although only relatively short kinetics were employed in this experiment, other studies have shown that neutralising antibodies can be detected for at least a year following a single immunisation of microencapsulated peptide or protein [457].

Protein antigens cannot be used for *in vitro* restimulation of certain lymph node cultures unless exogenous feeders are added, so most lymph node cultures were restimulated with the immunodominant Der p 1 epitope Der p 1;p111-139 instead of the whole protein. The lack of a proliferative response in most lymph node cultures may be attributed to kinetics. Most draining lymph nodes were removed at least a week after immunisation, by which time the antigen-specific lymphocytes may have left the site of immunisation and migrated to other lymphoid organs such as the spleen.

As described previously the oral delivery of PLG microparticles confers a number of advantages over systemic immunisation. PLG microspheres protect the

antigen from degradation [488] and are designed to increase antigen uptake by M cells [488, 561]. Certain parallels between the functions of M cells and macrophages have been drawn with respect to the uptake of particulate antigens [462].

In this study, the oral administration of microencapsulated Der p 1 particles did not elicit an immune response. This could be due to a number of reasons but can probably be attributed to insufficient priming. Although an intensive dosing schedule was employed this does not seem to have had an effect. One study suggests that only a tiny fraction (<1%) of ingested microparticles is actually taken up by the gut-associated lymphoid tissue [463], on the other hand, PLG microencapsulation is thought to enhance the particles' uptake by M cells [489, 490] because of strong adhesive interactions with gastrointestinal mucus and the brush-border glycocalyx [388]. This layer contains absorbed pancreatic enzymes and stalked intramembrane glycoprotein enzymes responsible for digestion [390]. The PLG microparticles maintain contact with the intestinal epithelium for extended periods of time and actually penetrate through and between cells. Strong biologically adhesive interactions delay the passage of microparticles through the gastrointestinal system. A study by Mathiowitz et al. suggested that some of the best materials for the construction of biologically erodable microparticles were polyanhydride copolymers of fumaric and sebacic acid which demonstrated higher biological adhesive properties than PLG [491].

There are plenty of studies to suggest that immune responses (mucosal and systemic), can be induced by utilising the oral route of immunisation [448, 466, 472, 492], although the dosing regimens vary greatly with multiple doses or combinations of oral and parenteral administrations. One study showed that an immune response could be detected after only one oral dose [492]. Repeated oral feeding of MEA Der p 1 in our study did not prime mice for a detectable immune response. In fact in order to induce antibody responses in orally fed mice, a subcutaneous boost with MEA Der p 1 was required in addition to the oral immunisation protocol. A combination of oral and subcutaneous immunisations of MEA Der p 1 did not enhance antibody responses above the levels observed for subcutaneous administration alone (results not shown).

There was however, the possibility that oral priming was inducing a tolerant state in the mice, and that this rendered them non-responsive rather than unprimed. To answer this question, mice were fed on three consecutive days with 42 mg MEA Der p 1 (containing approximately 300 μg Der p 1) or placebo and then challenged subcutaneously 28 days later with 50 μg Der p 1 in CFA. The mice were sacrificed 10 days later with no difference in proliferative responses being detected between control and experimental animals (figure 4.17). Therefore, this experiment suggested that oral administration of MEA Der p 1 did not induce a state of tolerance in the mice.

Serological data indicated that subcutaneous treatment with MEA Der p 1 was necessary to prime antibody responses. In agreement with other studies by this group, the predominant antibody isotype was IgG1. Many investigators have confirmed that for certain infectious diseases, the mucosal route is the most appropriate route of immunisation [474, 475, 488, 493]. It has been shown that several anatomically separate mucosal surfaces can be protected through the mucosal administration of a vaccine at a single site [494, 495]. Mucosal vaccination offers the added advantage that some degree of systemic immunity can be induced along with local responses, due to translocation of antigenic material from sub-epithelial compartments to systemic lymphoid organs such as the spleen. As sensitisation to Der p 1 occurs through the mucosa, it makes sense to adopt a vaccination protocol via the same route, which was why the intra-nasal route of administration was chosen. In addition, the nasal epithelium is more accessible to high molecular weight molecules than in other mucosa [496], and its mucus coating contains lower levels of proteolytic enzymes than in the gut [497]. Furthermore, it has been proposed that the nasal-associated lymphoid tissues (NALT) have a lymphoepithelium, which, like that in the intestinal mucosa, contains M-cells for selective antigen uptake [473, 498].

A previous study by Almeida *et al* in rats and rabbits showed that particles of average size 0.51 μm and 0.83 μm when administered intra-nasally could penetrate into the blood circulation [464]. Our particles ranged in size mostly from 1 – 10 μm but a small proportion of particles were <1 μm and a second study by Yan *et al* found that microparticles between 2.6 – 5.8 μm were very effective carriers of their chosen immunogen (ricin toxoid) [475].

With this in mind we decided to investigate whether intranasal administration of MEA Der p 1;p111-139 peptide before sensitisation could protect against the induction of allergic response. The hypothesis was that intranasal administration of MEA peptide would induce tolerance more readily and thus protect against a local challenge with Der p 1. Also, concerns about the potential for triggering IgE mediated inflammatory responses by using whole Der p 1, have prompted investigations on the use of T cell epitope peptides in therapy. Previous studies demonstrated that residues p111-139 of Der p 1 contained an immunodominant T cell epitope. Stimulation with a peptide covering this region induced lymphoproliferative responses in human T cell clones, and murine T cell lines obtained from Der p 1 immunised mice expressing the H2-b haplotype [499, 500]. The intranasal administration of peptide Der p 1;p111-139 to naive mice protected against the induction of Th1 mediated DTH responses, following immunisation with Der p 1 in CFA [501].

From the cytokine and antibody data, there is a possibility that tolerance in MEA peptide mice has been induced. The fact that Der p 1-specific IgE can still be detected though casts some doubt over tolerance induction. A study by Hurst and colleagues found that tolerance was established following the inhalation of small amounts of aerosolised OVA [437]. The characteristics of this tolerance were the absence of antigen-specific IgE *in vivo*, and both Th1 and Th2 cytokines upon Ag restimulation *in vitro*. Seymour *et al* found that the establishment of aerosol-induced IgE tolerance did not involve immune Th2 → Th1 deviation [502]. In addition, Hurst *et al* found that this form of tolerance was accompanied by high titres of antigen-specific IgG1. This fact supports our own study where MEA peptide-treated mice showed the highest levels of Der p 1-specific IgG1 (figure 4.28). Surprisingly, mice treated with peptide alone had much lower levels of IgG1 and cytokine levels were markedly (but not statistically) higher than MEA peptide mice.

The eosinophil infiltration results were striking although again, were not statistically significant (figure 4.26). Mice administered MEA encapsulated peptide intranasally had a marked reduction in the number of lung eosinophils after inhalation of the Der p 1 allergen. Mucosal tolerance is linked to the degree to which the $CD4^+$ T cell population is initially activated, since the intranasal administration of

peptide alone, failed to protect against the induction of eosinophilia. As discussed earlier, antigen concentration and TCR affinity are critical in determining the strength of initial activation signals that precede T cell tolerance [136, 503]. The data suggests that by using MEA peptide, the concentration of peptide released systemically was increased, thus strengthening the initial TCR mediated activation signal. Studies involving the intranasal administration of whole antigen, demonstrated that the induction of peripheral T cell tolerance was accompanied by modified B cell responses, with reductions in specific IgE and changes in the ratio of IgG1 to IgG2a levels in sera [146, 504]. In this study, the intranasal administration of a microencapsulated T cell epitope peptide suppressed local Th2 dependent inflammatory responses in the airways but did not greatly influence B cell dependent responses such as antibody levels in sera. A number of studies in animal models of asthma demonstrate that the typical processes of asthma such as eosinophil infiltration of the bronchial mucosa and BHR are elicited independently of the presence of B cells and IgE Ab's, but are dependent on the presence of T cells [505-507]. Failure to significantly downregulate allergen induced eosinophilia following mucosal delivery of peptide to sensitised mice, suggests that it may be more difficult to suppress established Th2 mediated responses, by tolerance induction. This view is supported by a recent publication demonstrating that ongoing Th2 responses in the lungs of mice prevented induction of tolerance to aerosolised OVA [437].

Tolerance can be mediated by a number of mechanisms. One mechanism is that of linked suppression which is thought to account for the loss of responsiveness to all epitopes within a protein, following the induction of T cell tolerance using a single immunodominant epitope. This form of bystander suppression may be mediated via an antigen non-specific cognate interaction with APCs [508], possibly involving signalling through notch ligands and their receptors [342] and / or through the production of immunosuppressive cytokines such as IL-10 and TGF-β. IL-10 has been shown to be critical in the induction of mucosal tolerance to a Th1 response to autoantigen [509]. In addition, an increase in IL-10 production by peripheral $CD4^+$ T cells has been shown in clinical studies following allergen specific immunotherapy (SIT) [510].

In this study, an increase in IL-10 production was not observed by antigen stimulated cells from the draining lymph nodes of mice treated intranasally with MEA peptide. However, there was a large decrease in eosinophilia (figure 4.26) in the MEA peptide group which was paralleled by a reduction in IL-5 (figures 4.20 and 4.24) - critical to the differentiation and survival of eosinophils. Although we were unable to detect an increase in IL-10, it is possible that IL-10 induced tolerance in the antigen reactive $CD4^+$ Th2 population, may have occurred during antigen presentation in the draining lymph nodes, resulting in a loss of localised IL-5 production and consequently a fall in airway eosinophil levels.

Results from the placebo mice were quite intriguing. The level of IL-5 (figures 4.2 and 4.24) and IL-13 production (figures 4.21 and 4.24) suggested that a Th2 response was being induced. However, the experiment was only able to be undertaken once and only four mice were tested. The largest inter-group variation was exhibited by the placebo mice therefore, it would be preferable to repeat the experiment before any firm conclusions can be drawn on the effect of placebo microparticles on Th2 induction.

Recent evidence suggests that the induction of mucosal tolerance may be dependent on IL-10 production by pulmonary dendritic cells [511]. IL-10 is a critical growth factor for a population of antigen specific $CD4^+$ $CD45RB^{low}CD25^+$ T regulatory type 1 cells (Tr1), which are thought to be central to the induction and maintenance of peripheral tolerance [512, 513]. Cottrez *et al* showed that the transfer of Tr1 T cell clones but not Th1 or Th2 clones, at the time of OVA immunisation inhibited antigen specific serum IgE responses [514]. Although IL-10 was originally described as a murine Th2 factor, it is now recognised that this cytokine inhibits Th2 mediated responses, including airway eosinophilia [515]. The ability of IL-10 to suppress DC maturation may be central to the induction of T cell tolerance and immunoregulation by Tr1 cells [516, 517]. A recent study by Zuany-Amorim and colleagues showed that the treatment of mice with SRP299, a killed *Mycobacterium vaccae*-suspension, gives rise to allergen-specific $CD4^+CD45RB^{lo}$ regulatory T cells, which confer protection against airway inflammation [583]. This specific inhibition was mediated through IL-10 and TGF-β, as antibodies against IL-10 and TGF-β completely reversed the inhibitory effect of $CD4^+CD45RB^{lo}$ T cells.

Even though preceding MEA Der p 1 experiments suggested that the induced immune response was largely Th2-orientated, it was decided to give MEA Der p 1 to mice with an established Th2 response, as this most closely mimicked the real life situation where most immunotherapies are given to patients with established allergic disease. Mice were primed for a Th2 response with a number of systemic immunisations of Der p 1 (without adjuvant), in order to establish more physiological conditions, perhaps comparable to exposure in man. Mice then received five doses of MEA Der p 1 intranasally before receiving three tracheal instillations of Der p 1, to determine whether intranasal administration of the antigen could tolerise an existing Th2 response to subsequent airway challenge with Der p 1. It was found that the i.n. administration of MEA Der p 1 actually increased Th2 priming and in fact, exacerbated the allergic response as exhibited by the lung eosinophil data. Similar results were observed when Der p 1 alone was given intranasally. It seems that our established Th2 response was far too strong to be down-regulated. This is supported by the results of Hurst and colleagues who found that the establishment of normal IgE tolerance to inhaled OVA Ag could be subverted by strong local Th2 responses but not Th1 responses, to unrelated antigens or pathogens in the lung, and that this prevention was largely the consequence of local IL-4 production immediately before inhalation of OVA [437]. An intranasal KLH challenge to sensitised KLH/alum before the primary exposure of mice to OVA by aerosolisation resulted in the sensitisation, not tolerance of mice to OVA. Likewise, infection of mice with antigens such as *Nippostrongylus brasiliensis* or exposure to *A. fumergatus* extract, both of which produce vigorous Th2 responses, prevented the establishment of IgE tolerance to aerosolised OVA. There is no evidence from other groups that the encapsulation of any antigen in PLG microparticles can down-regulate a Th2 response.

Chapter 5

Chitosan

5.1 Introduction

Chitosan is a polysaccharide composed of two subunits, D-glucosamine and N-acetyl-D-glucosamine linked together by β(1,4) glycosidic bonds. It is obtained industrially by hydrolysing the aminoacetyl groups of chitin. Chitin is one of the most abundant polysaccharides in nature, second only to cellulose. It is a structural component of the exoskeleton of crustacea and insects. After the deacetylation of chitin, the resulting chitosan is dissolved in acid, filtered, washed and dried to give amine free chitosan. The chemical formula of chitosan is $(C_6H_{11}O_4N)_n$.

Chitosan has already been used in a wide array of applications – personal care products (hair treatment, skin care, moisturiser, wound healing), food products (wine and juice clarification, protective fruit coating, hypercholosterolemic behaviour), for clarification and waste management (treatment of sewage, sludge and brewers' waste, chelation of toxic heavy metals and radioactive materials), in agriculture (seed coating, as a flocculating agent, as a controlled release agent for pesticides and herbicides), for biotechnology products (to separate and purify biologicals and to immobilise enzymes and cells) and in pharmaceuticals [518].

Chitosan is soluble in acidic solutions but insoluble at pH > 6.5 and in most organic solvents. This property of chitosan is disadvantageous for biomedical applications because at physiological pH (7.4), most chitosans precipitate from solution and consequently become ineffective. In recent years, chitosan has been manipulated in order to increase its solubility over a wider pH range. Several derivatives of chitosan have been synthesised among which is N-trimethyl chitosan chloride (TMC). It was found that the greater the degree of trimethylation, the greater the solubility of the polymer at neutral pH values [519].

Chitosan is considered to be a biocompatible, biodegradable and non-toxic polymer [520]. It was found not to have a damaging effect on nasal membranes in

rats [521] and also, is non-toxic in humans as studies have shown that when administered intra-nasally, only transient decreases in mucociliary transport rates were detected *in vitro*, leaving no permanent damage to this defence system [522]. However, one group studied the effects of chitosan on a murine melanoma cell line (B16F10) and rat erythrocytes and found that cell viability was compromised and haemoglobin release was increased in a concentration-dependent way [523].

The mechanism of action of chitosan is believed to be a combination of bioadhesion and an ability to widen the tight junctions between epithelial cells in a transient fashion. The fact that chitosan is a cationic macromolecule, means that it can interact with anionic components (e.g. sialic acid) of the glycoproteins on the surface of epithelial cells. In addition, the interior of tight junctions is highly hydrated and contains fixed negative charges. A change in the relative concentration of specific ion species within the tight junction could result in an alteration of tight junction electrical resistance which, in turn, could lead to its opening [524].

As discussed previously in this thesis, the nasal mucosa is an attractive site for the non-parenteral delivery of proteins and peptides for the induction of mucosal immunity or tolerance. The benefits of nasal delivery include ease of administration and patient acceptability however, this route of delivery has a number of drawbacks. First, the nasal mucosa provides a substantial barrier to the free diffusion of macromolecules [525]. Second, the enzymatic activity present in nasal secretions can limit protein delivery [526]. Third, in humans, the surface area of the nasal mucosa is only ~ 150 cm^2 [527], limiting the amount of protein that can be absorbed from one application. Finally, the typical residence time of a protein delivered to the nasal mucosa in only 15-30 minutes due to rapid ciliary clearance [528]. A possible strategy to circumvent this, is to prolong the contact between the protein/peptide and the mucosa. Chitosan acts as a bioadhesive system by forming a gel-like structure at the contact of the mucus. However, the use of chitosan in nasal formulations still remains under investigation with relatively few studies published in the literature.

The first published study based on a chitosan nasal delivery system containing a model drug (insulin) was by Illum and colleagues [529]. Their main goal was the enhancement of the systemic bioavailability of insulin after intranasal instillation in rats and sheep. The authors showed that a concentration of 0.5%

polymer was sufficient to vastly improve plasma insulin levels. More recently, insulin-loaded chitosan nanoparticles were used in a rabbit model and were found to enhance the nasal absorption of insulin to a greater extent than an aqueous solution of chitosan and insulin [530].

Bacon *et al* investigated the ability of chitosan to augment the immunogenicity of influenza vaccines given intranasally [531]. Groups of mice were vaccinated three times intranasally with 10 μg of purified influenza B/Panama virus surface antigens (PSAs) either alone or mixed with chitosan. Parenteral immunisation with B/Panama PSA and Alhydrogel elicited high titres of anti-B/Panama antibodies in serum but a very poor respiratory anti-B/Panama IgA response. In contrast, intranasal immunisation with PSA plus chitosan stimulated very strong local and systemic anti-B/Panama responses. The mean serum IgG titres and the HAI (haemagglutinin inhibition) titres of the PSA-plus-chitosan-immunised mice were significantly greater ($P < 0.05$) than those of the PSA intranasal immunised mice.

Chitosan has also been investigated as a candidate vector for DNA delivery [532-534]. In one study, low molecular mass chitosans were found to complex DNA more effectively than poly (L-lysine) was able to and also provided protection against nuclease degradation [533]. In another study, chitosan-DNA (encoding a peanut allergen gene) nanoparticles were orally administered to mice. These mice produced secretory IgA and serum IgG2a and showed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE, plasma histamine and vascular leakage [534].

In conclusion, the majority of current studies into chitosan, seem to be focussed on enhanced drug or DNA delivery, especially across mucosal surfaces (e.g. nasal or gut).

As discussed earlier, Der p 1 is a major target allergen of the human immune response to house dust mite in allergic asthmatics. The use of Der p 1 as an intact molecule in immunotherapy via the airways has inherent limitations due to the triggering of mast cell degranulation and potentially life-threatening anaphylaxis. A number of clinical studies have investigated the use of peptides in conventional therapy using the systemic route of delivery, as a means of avoiding the risk of

triggering inflammatory responses following the cross-linking of FcεR's on effector cells by IgE-allergen complexes [535].

In this study, the intranasal administration of a peptide encoding an immunodominant T cell epitope of Der p 1 was investigated to see whether peripheral $CD4^+$ T cell responses and airway eosinophilia could be suppressed in a murine model of pulmonary inflammation. Previous studies have shown that intranasal administration of Der p 1;p111-139 protected against the induction of systemic Th1-mediated DTH responses in mice immunised with Der p 1 in CFA. Antigen-specific T cell tolerance correlated with loss of proliferation, IL-2 production and ability to provide cognate help for antibody production, following stimulation *in vitro* [501].

The Der p 1;p111-139 was adsorbed to chitosan prior to administration in order to maximise the passive diffusion of peptide across the intercellular barriers, formed by the tight junctions. This maximisation was critical in order to reach the TCR activation threshold for tolerance induction. Thus, the delivery of peptide adsorbed to chitosan could facilitate tolerance induction by increasing the level of peptide released systemically [536, 537].

5.2 Investigating whether Chitosan promotes the induction of tolerance.

Protocol

Mice (female, C57BL/6, 6-8 weeks old) were immunised on days 0, 1, 2 and 3 with either:

1. 20 µl 0.25% w/v chitosan i.n. (n = 15)
2. 100 µg Der p 1;p111-139 in 20 µl PBS i.n. (n = 15)
3. 100 µg Der p 1;p111-139 in 20 µl 0.25% w/v chitosan i.n. (n = 15)

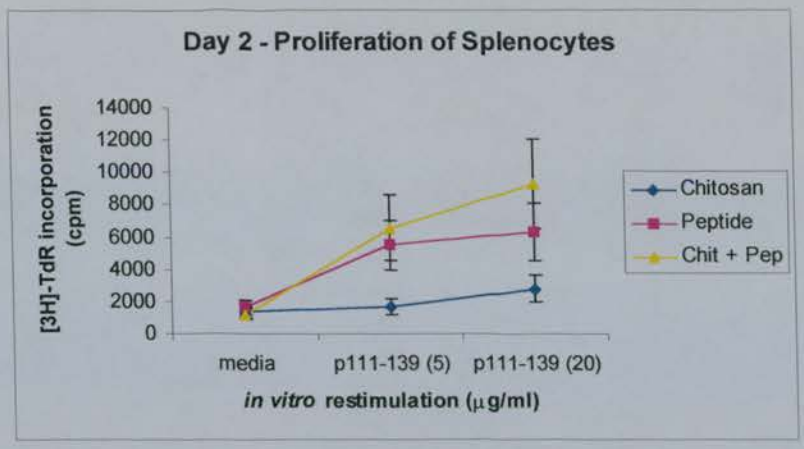
For i.n. immunisations, mice were lightly anaesthetised with halothane, and 20 µl of sample (10 µl per nostril) was applied to the external nares with a micropipette (Gilson). Five mice from each group were sacrificed on days 2, 4 and 8 after the last i.n. immunisation. Spleen and cervical lymph nodes were removed from each mouse. Pooled cervical lymph node cells and splenocytes from individual mice were restimulated *in vitro* with either media alone, or media supplemented with 5 and 20 µg/ml Der p 1;p111-139. 20 µg/ml of haemagglutinin peptide (HA) – an unrelated antigen was used as a specificity control, and α-CD3/CD28 (5 µg/ml:2.5 µg/ml) was included as a positive control. Supernatants were removed at 72 hours for cytokine determination. The cells were pulsed at 72 hrs with [^{3}H]-thymidine for a further 18 hours when the cells were harvested to determine the extent of proliferation.

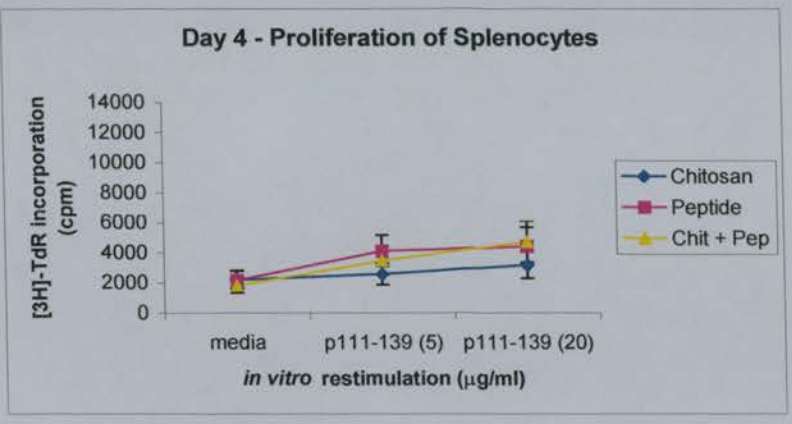
Results

The aim of this study was to determine whether the intranasal administration of an immunodominant T cell epitope of Der p 1;p111-139 adsorbed to chitosan, protected against the induction of Th2 mediated airway inflammatory responses to the Der p 1 allergen in a murine model of pulmonary inflammation.

The first question to address, was whether Der p 1;p111-139 adsorbed to chitosan and delivered intranasally, was able to prime an immune response and to determine the qualitative nature of that response. For this purpose, mice were administered an equivalent of (3 x 100 μg) Der p 1;p111-139 intranasally on 3 consecutive days. After 2 days, 4 days and 8 days, lymphocytes were isolated from the cervical lymph nodes draining the nasal cavity and from the spleen, which were then stimulated with increasing doses of peptide. The results shown are from one experiment.

At 2 days, $CD4^+$ lymphocytes from peptide and chitosan/peptide mice were hyperresponsive to *in vitro* restimulation with Der p1;p111-139 peptide, with dose dependent increases in proliferation and production of IL-5 and IFN-γ (figures 5.1 – 5.8). Closer investigation of the cytokine recall response revealed that at equimolar concentrations of peptide, T cells isolated from chitosan-peptide treated mice exhibited a change in T cell effector function from a Th2/Th0 to a Th1/Th0 phenotype, compared to the peptide treated mice, which was accompanied by an increase in the ratio of IFN-γ to IL-5 production. This was consistent at 5 μg/ml and 20 μg/ml Der p 1;p111-139, indicating that this was not due to differences in TCR activation thresholds. By day 4 and certainly day 8, T cells obtained from all groups of mice were hyporesponsive to in vitro restimulation with Der p 1;p111-139 peptide, with a loss in both proliferative and cytokine responses. Mice treated with chitosan alone did not show any T cell activation. There was no response to *in vitro* restimulation with HA by any of the experimental groups, proving that the proliferative responses observed after restimulation with Der p 1 or its related peptides were antigen specific.





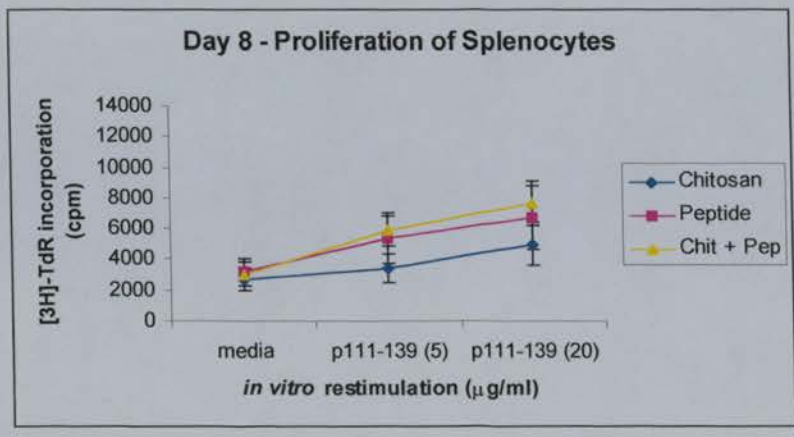
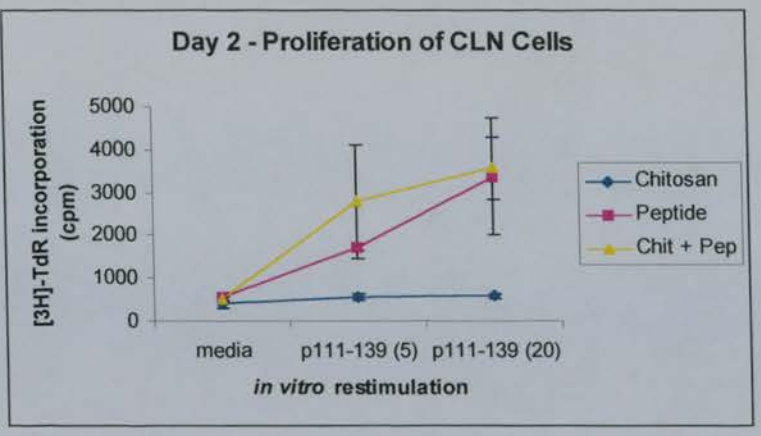
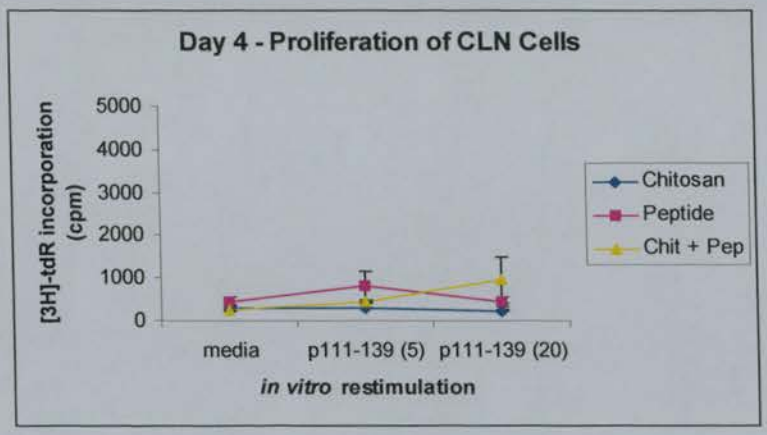


Figure 5.1 – Investigating whether chitosan promotes tolerance induction. Proliferation of splenocytes.
Single cell suspensions were prepared from individual spleens which were then cultured ($5x10^5$/well) with media alone or media supplemented with 5 and 20 μg/ml Der p 1:p111-139. Proliferation was measured at 90 hours after pulsing with thymidine at 72 hours.
Mean +/- SE is shown.





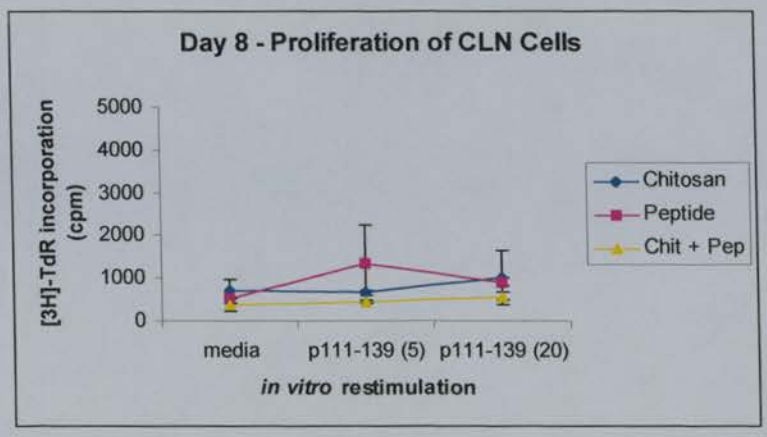
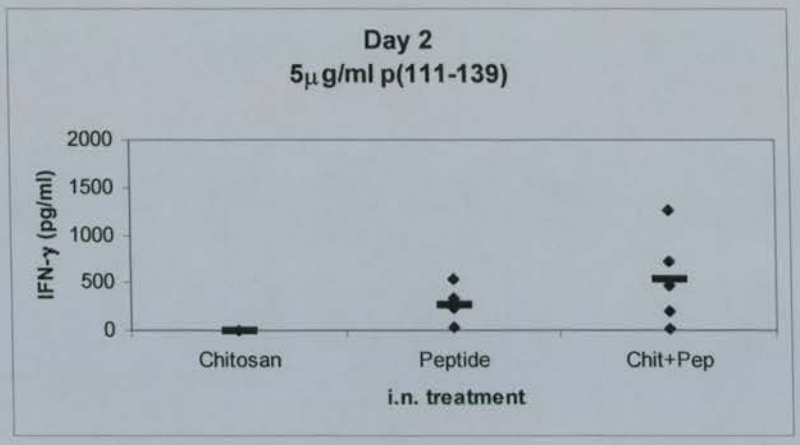
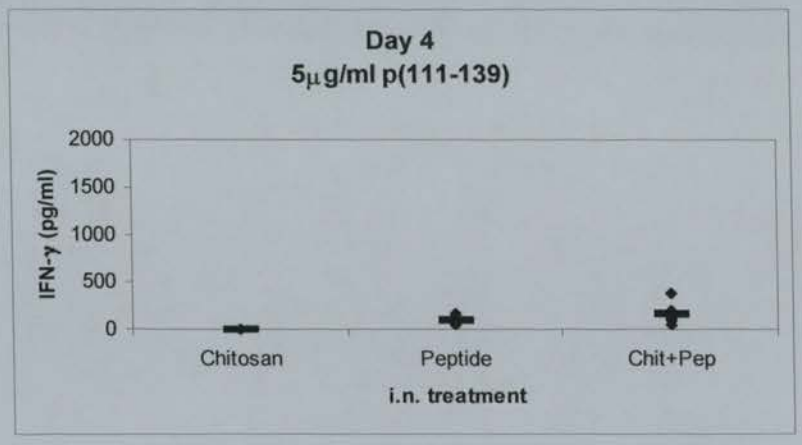


Figure 5.2 – Investigating whether chitosan promotes tolerance induction. Proliferation of CLN cells.

Single cell suspensions were prepared from pooled cervical lymph nodes which were then cultured ($5x10^5$/well) with media alone or media supplemented with 5 and 20 μg/ml Der p 1;p(111-139). Proliferation was measured at 90 hours after pulsing with thymidine at 72 hours.

Mean +/- SE is shown.





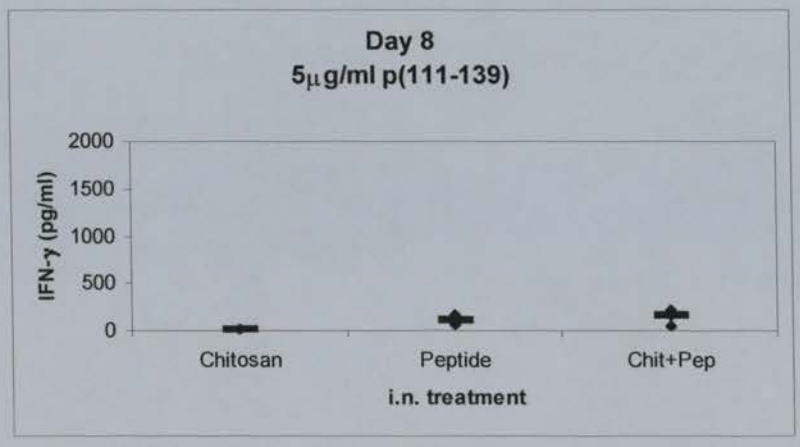


Figure 5.3 – Investigating whether chitosan promotes tolerance induction. IFN-γ production by splenocytes after restimulation with 5 μg/ml Der p 1 p(111-139).

Supernatants were removed at 72hours and frozen at -20°C until analysis by ELISA. Individual mice are represented by diamonds, mean values within each group are represented by bars.

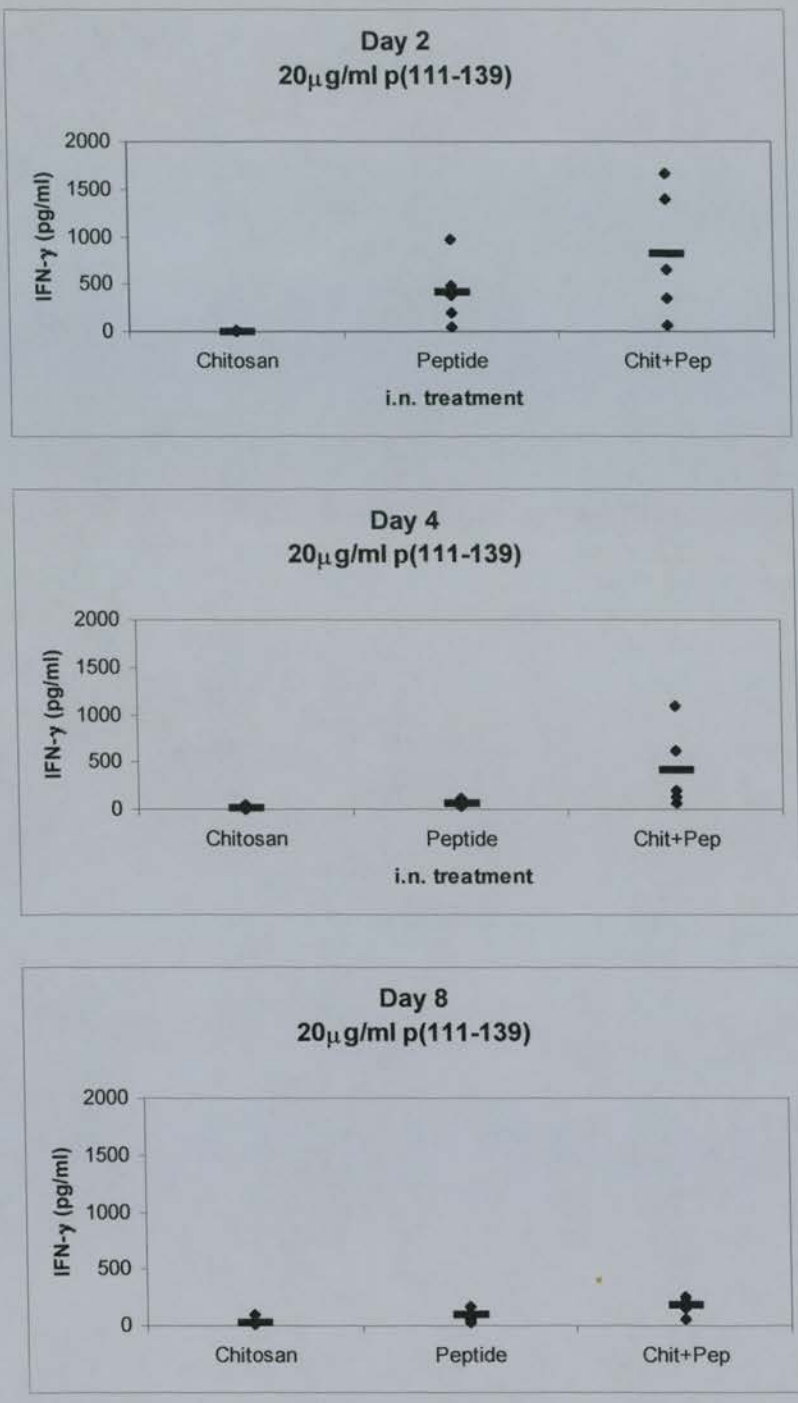


Figure 5.4 – Investigating whether chitosan promotes tolerance induction. IFN-γ production by splenocytes after restimulation with 20 μg/ml Der p 1 p(111-139).

Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA. Individual mice are represented by diamonds, mean values within each group are represented by bars.

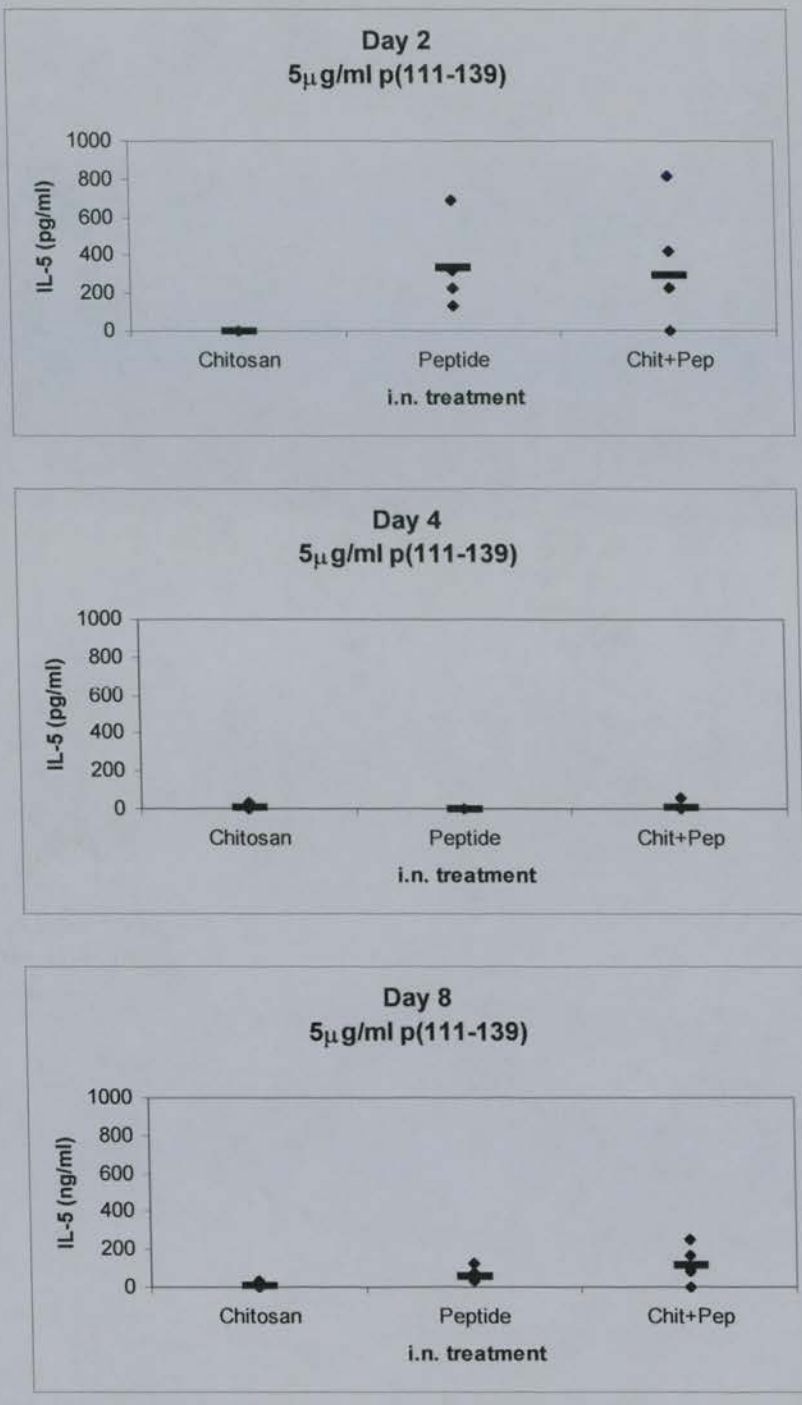


Figure 5.5 – Investigating whether chitosan promotes tolerance induction. IL-5 production by splenocytes after restimulation with 5 μg/ml Der p 1 p(111-139).

Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA. Individual mice are represented by diamonds, mean values within each group are represented by bars.

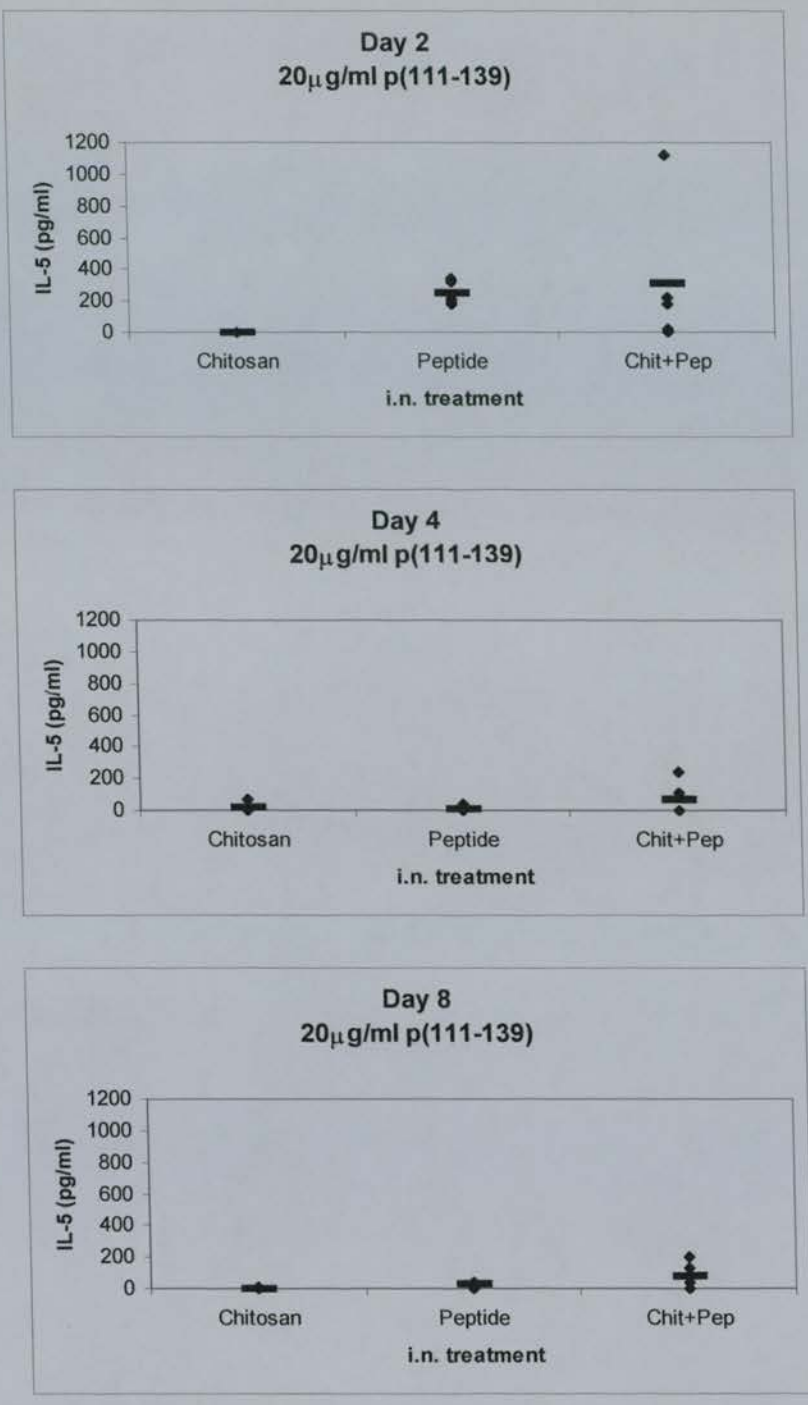


Figure 5.6 – Investigating whether chitosan promotes tolerance induction. IL-5 production by splenocytes after restimulation with 20μg/ml Der p 1 p(111-139).

Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA. Individual mice are represented by diamonds, mean values within each group are represented by bars.

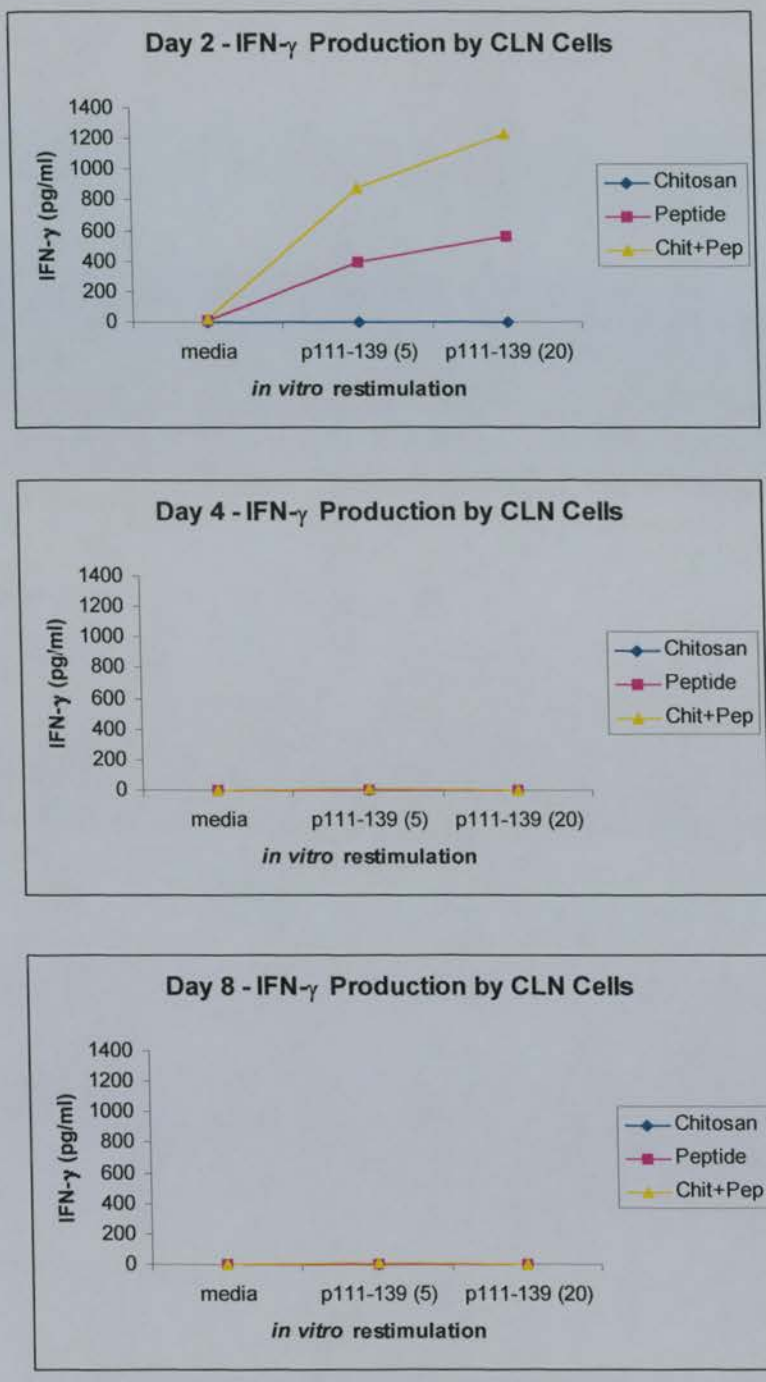


Figure 5.7 – Investigating whether chitosan promotes tolerance induction. IFN-γ production by CLN cells.
Single cell suspensions were prepared from pooled cervical lymph nodes and restimulated in vitro with media alone or media supplemented with 5 and 20 μg/ml Der p 1;p(111-139). Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA.

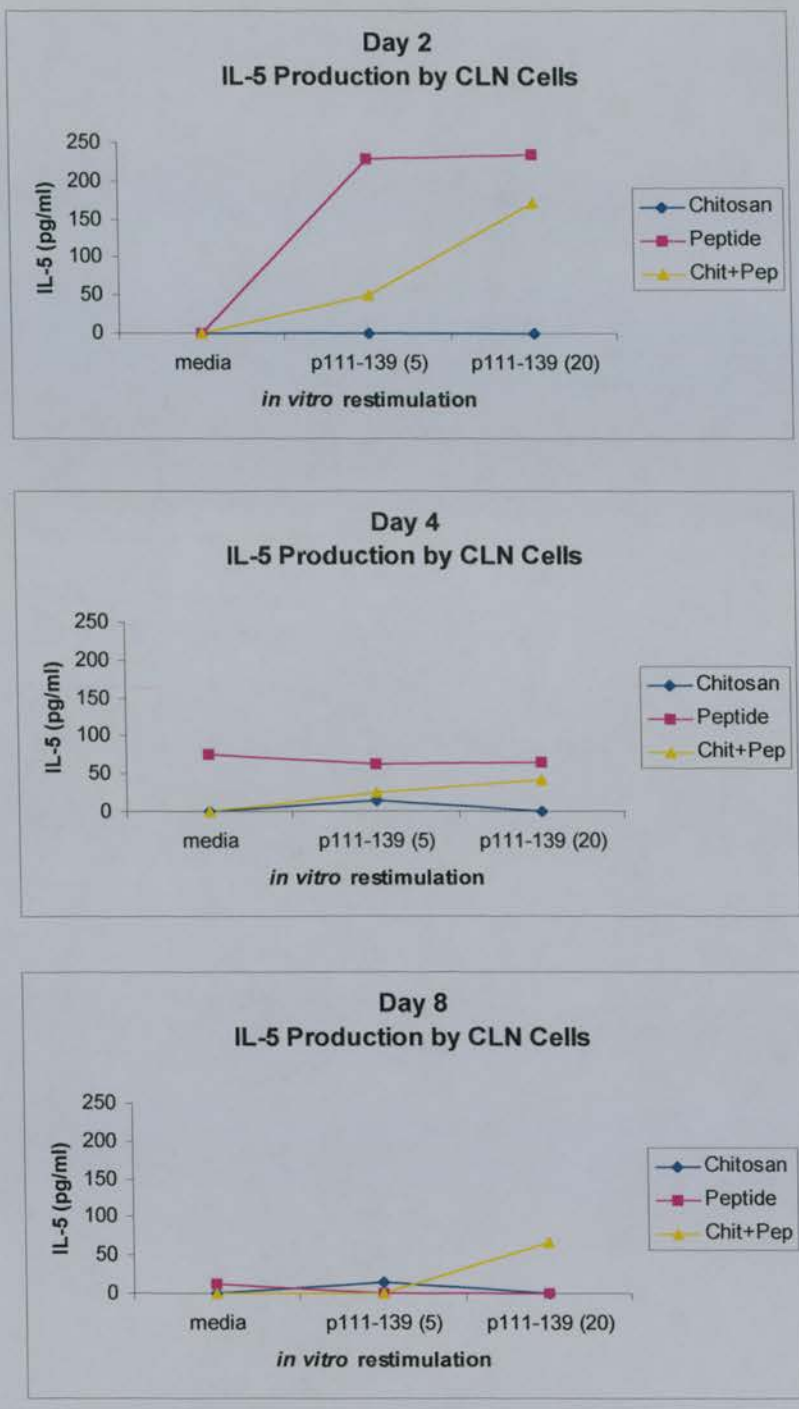


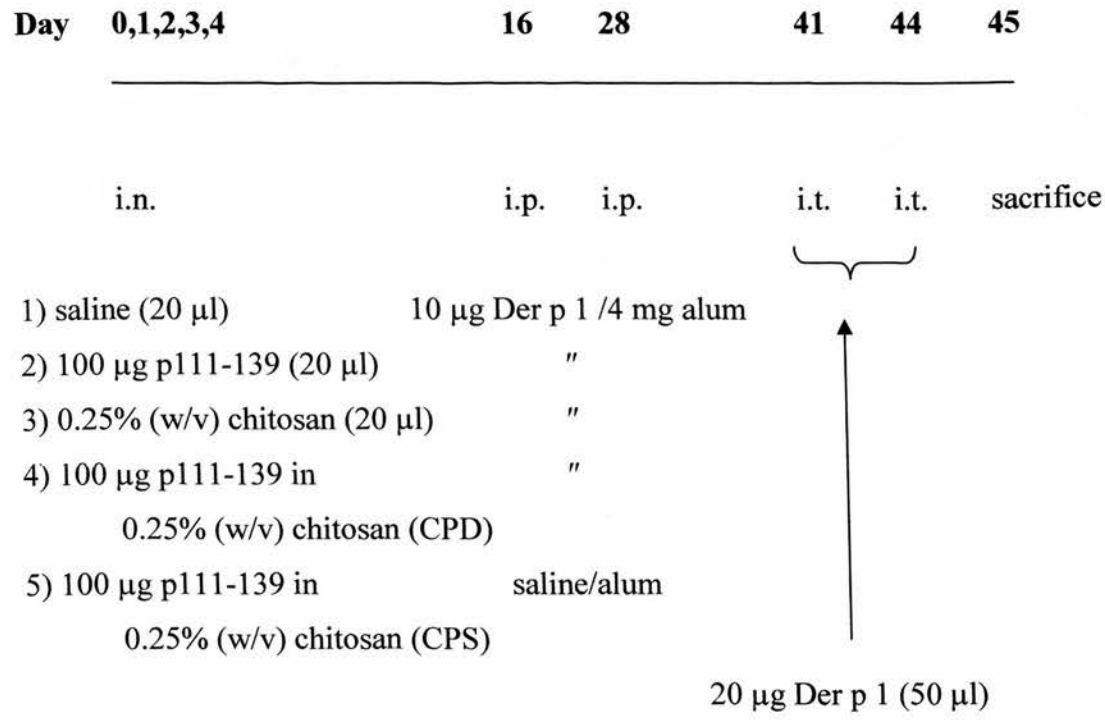
Figure 5.8 – Investigating whether chitosan promotes tolerance induction. IL-5 production by CLN cells.
Single cell suspensions were prepared from pooled cervical lymph nodes and restimulated in vitro with media alone or media supplemented with 5 and 20 μg/ml Der p 1;p(111-139). Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA.

5.3 Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2-mediated responses in a murine model of pulmonary inflammation.

The previous study was extended by investigating whether the i.n. administration Der p 1;p111-139 could prevent the induction of peripheral $CD4^+$ T cell responses and airway eosinophilia when adsorbed to chitosan, in a murine model of pulmonary inflammation. As discussed earlier in this chapter, chitosan is thought to enhance the passage of peptide across the mucosa through the tight junctions thus leading to increased TCR activation and potentially enhance tolerance induction.

To address this hypothesis, mice were immunised i.n. on five consecutive days with Der p1;p111-139, adsorbed to chitosan or saline alone, chitosan alone, and Der p 1:p111-139 alone (protocol shown in detail on next page). After 12 days the mice received two i.p. injections of Der p 1/alum 12 days apart. The mice were rested for 13 days before receiving two intra-tracheal instillations of Der p 1 three days apart. The mice were sacrificed 24 hours after the last i.t. challenge. Lung lavage was immediately performed *in situ* after death in order to obtain BAL fluid for cytospins and cytokine determination. Whole lungs were removed to determine the extent of eosinophil infiltration, and sera was taken to determine the presence of Der p 1-specific IgG1, IgG2a and IgE antibodies. Tracheal lymph nodes were removed and pooled within groups along with individual spleens. Single cell suspensions were prepared from each mouse for *in vitro* restimulation with media alone or media supplemented with 20 μg/ml Der p 1, p(111-139), p(111-119), p(113-127), OVA peptide (an unrelated antigen) and α-CD3/CD28 (as a positive control, 5 μg/ml:2.5 μg/ml respectively).

Protocol



For each experimental group n=5 C57BL/6 female mice (6-8 weeks old).

CPD = Der p 1 adsorbed to chitosan followed by Der p 1 in alum

CPS = Der p 1 adsorbed to chitosan followed by Der p 1 in saline

Results

This study investigated whether the i.n. administration of peptide adsorbed to chitosan could lead to tolerance and a loss of cytokine production or alternatively, facilitate the differentiation of an effector T cell population with a Th1/Th0 phenotype and thereby, prevent the induction of allergic inflammation.

Effect of intranasal peptide administration on proliferative responses.

Figure 5.9 shows the proliferation of splenocytes in response to various stimuli. The major difference between groups was observed for Der p 1 and Der p 1;p111-139 *in vitro* restimulation. Proliferation of splenocytes from the chitosan-treated mice was at similar levels to saline treated mice and higher than the other groups. The stimulation indices in table 5.1a show little difference between the other groups. For the other stimuli, proliferation was barely above background levels and there were no clear differences between groups. As in the previous experiment there was no response to the specificity control (OVA peptide), proving that the responses observed were antigen-specific.

Cytokine production by allergen reactive T cells isolated from mice treated prophylacticaly by intranasal peptide administration.

Levels of the cytokines IL-5, IL-13, IL-10 and IFN-γ in culture supernatants were determined by ELISA. A summary of cytokine production in splenocytes after restimulation with 20 μg/ml Der p 1 is shown in table 5.1b. IL-5 production by splenocytes after restimulation with Der p 1; p111-139 and p113-127 is shown in figure 5.10. The largest differences can be observed after Der p 1 restimulation. There was a large amount of inter-group variation, thus, none of the data was statistically significant. However, certain trends could be observed. There was a 35% reduction in IL-5 production by splenocytes from the chitosan/peptide mice compared to the saline control. However, IL-5 production from the peptide alone mice was not suppressed (↑50% on saline mice). Restimulation with Der p 1;p111-139 and Der p 1;p113-127 resulted in a similar pattern of IL-5 production from all experimental groups. However, levels of production were decreased two-fold when restimulated with Der p 1;p111-139 and Der p 1;p113-127 compared to whole

Der p 1.

IL-13 production by splenocytes mirrored IL-5 production and is shown in figure 5.11. Although, IL-13 production by the peptide/chitosan mice was only 8% less than the saline controls, the peptide mice had an elevated production of 103%.

IFN-γ production by splenocytes was only evident with Der p 1 restimulation (figure 5.12). Saline-treated mice produced the most IFN-γ with chitosan-treated mice producing the least. IFN-γ production was also low in the chitosan/peptide mice which was considerably less (>50%) than the peptide-treated mice.

IL-10 production by splenocytes is shown in figure 5.14. Again, it is restimulation with Der p 1 that leads to the largest production of cytokine. The results are similar to IL-5 and IL-13 production with the chitosan/peptide mice producing 31% less IL-10 than the saline controls, and the peptide mice producing 23% more.

Figure 5.14 shows the IL-5 and IL-13 production from tracheal lymph node cells after various *in vitro* stimuli. In contrast to systemic responses, the cytokine response of Der p 1 reactive T cells isolated from the draining lymph nodes of the chitosan/peptide mice, 24 hours after challenge of the airways with Der p 1, were not inhibited as compared to the saline control group. Although the cytokine response from the chitosan/peptide mice was not inhibited, cytokine production by the peptide-treated mice was markedly increased (5-fold for IL-5 and 7-fold for IL-13). This increase in cytokine production observed in the draining lymph nodes rather than the spleen indicated that the intranasal administration of soluble peptide to naïve mice prior to sensitisation, primed localised Th2 responses. These differences were not so apparent with the other *in vitro* stimuli.

Therefore, it appears that a dichotomy exists in the cytokine profiles of allergen reactive T cells from local versus systemic sites in the chitosan/peptide mice. To investigate whether localised tolerance in the lymph nodes draining the sites of allergen exposure was maintained by active immunoregulation, levels of the immunosuppressive cytokine IL-10 were determined (figure 5.15). An increase of 100% in the level of IL-10 production by Der p 1 reactive T cells isolated from the tracheal lymph nodes of chitosan/peptide mice (compared to saline controls) was observed compared to a decrease of 31% in systemic IL-10 production (table 5.1b). T

cells isolated from the draining lymph nodes of peptide-treated mice showed an increase in IL-10 production of 70% on control mice but this level of production was insufficient to suppress eosinophilia (figure 5.16). This may be explained by the larger IL-5:IL-10 ratio of the peptide mice (8) compared to the peptide/chitosan mice (2) (table 5.1c).

Effect of intra-nasal peptide administration on eosinophil levels in Der p 1 allergen challenged airways.

The infiltration of eosinophils into the lungs was investigated by using the eosinophil peroxidase (EPO) assay and is shown in figure 5.16. This assay has limitations for the quantitation of eosinophilia and large inter-group variation can often be attributed to: lavage technique, weight of lungs (varies due to dryness of lungs) and loss of material to the homogenisation process. Thus, there was little difference in eosinophil infiltration between the four groups of mice. All groups have a level of eosinophilia of approximately 10,000 eosinophils/gram lung. The preparation of cytospins from the BAL fluid can also be used to assess the extent of eosinophil infiltration. This assay is considered less prone to the variables affecting the EPO assay. However, results mirrored that of the EPO assay with chitosan/peptide mice exhibiting similar levels of eosinophilia to the saline controls (figure 5.16).

The BAL fluid was also used to determine IL-5 and IL-4 levels. There was a 65% reduction in IL-5 and a 73% reduction in IL-4 from the chitosan/peptide mice compared to the saline controls. However, mice treated with chitosan alone also exhibited similar reductions in cytokine production (figure 5.17).

Effect of intranasal peptide treatment on antigen specific immunoglobulin levels induced following systemic immunisation.

To investigate whether the tolerisation of T cells reactive with an immunodominant region of Der p 1, by the intranasal administration of the peptide Der p 1;p111-139 was sufficient to inhibit antibody production following immunisation with the intact Der p 1 allergen, IgG1, IgE and IgG2a production was measured. Figure 5.18 shows the Der p 1-specific IgG1, IgG2a and IgE levels in

sera. By far the most abundant antibody was IgG1. The chitosan/peptide mice showed a decrease of 28% in IgG1 production compared to saline controls, however, the peptide treated mice showed a reduction of 74%. In contrast, IgE levels were not reduced. Chitosan/peptide mice showed an increase of 170% compared to saline controls ($P < 0.05$) with peptide treated mice showing an increase of 55% in IgE production. Levels of IgG2a were almost identical in all experimental groups. The increase in IgE production may be due to the non-specific inhibitory effect of chitosan on IFN-γ production, since IFN-γ counterbalances the development of Th2 responses and production of IL-4, a cytokine critical for induction of IgE. The failure to inhibit antibody production, in spite of significant reductions in Der p 1 reactive T cell effector functions, may be due to the emergence of T cells reactive with minor epitopes of Der p 1. Also, the memory response could be independent of the T helper cell help.

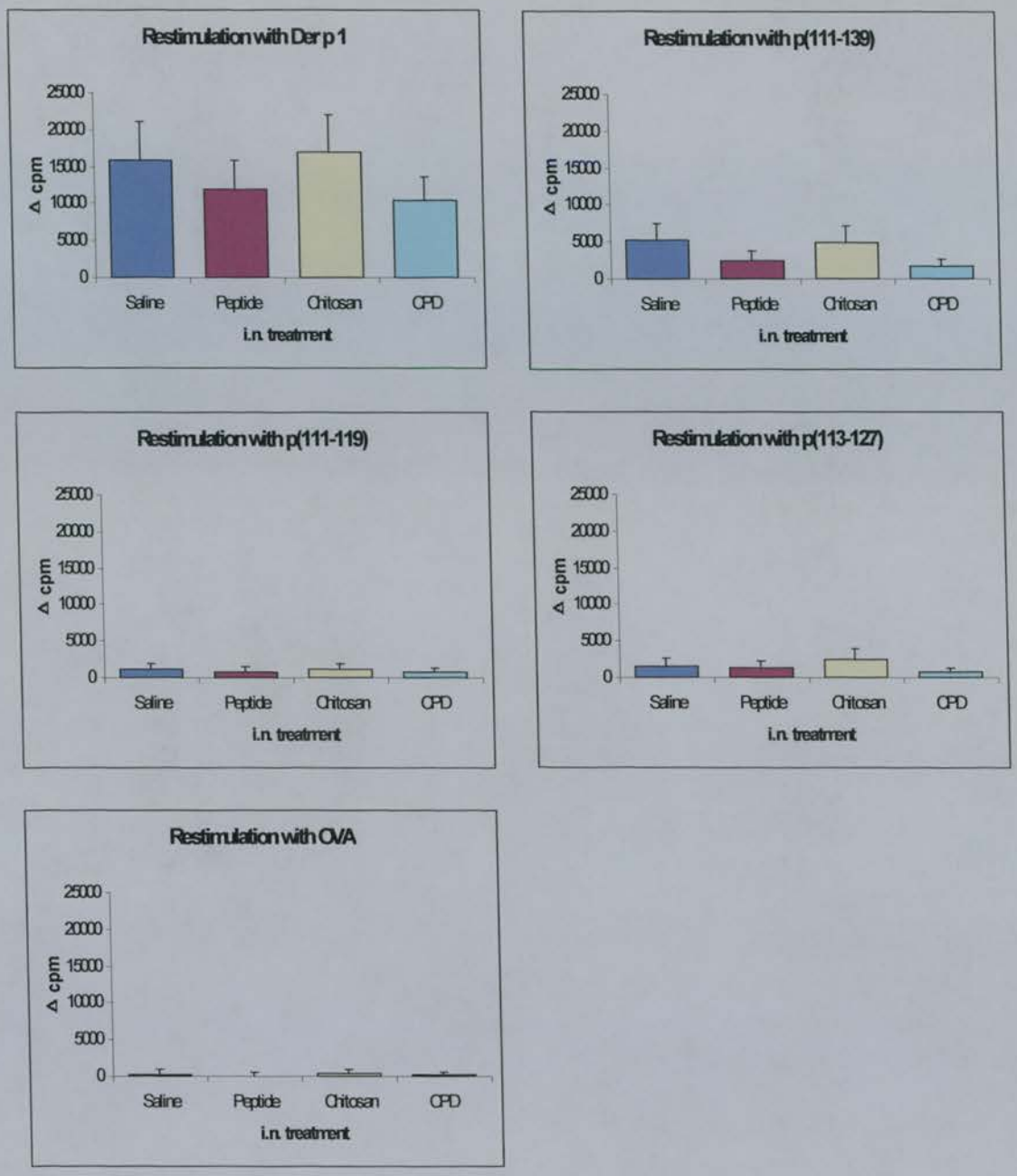


Fig. 5.9 – Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.
Proliferation of splenocytes.
Single cell suspensions were prepared from whole spleens which were cultured with media or media supplemented with 20 μg/ml Der p 1, 10 μg/ml Der p 1;p111-139, 10 μg/ml Der p 1;p111-119, 10 μg/ml Der p 1;p113-127 or 10 μg/ml OVA.
Proliferation was measured at 90 hours after pulsing with thymidine at 72 hours.
CPD = chitosan/peptide-treated mice
Mean +/- SE is shown.

Table 5.1a Proliferation of Splenocytes – Stimulation Indices

		In vitro restimulation with:					
		media	**Der p 1**	**p111-139**	**p111-119**	**p113-127**	**OVApep**
Intranasal treatment	**Saline**	1.0	9.3	3.8	1.5	1.8	1.1
	Peptide	1.0	9.1	2.6	1.5	1.9	1.0
	Chitosan	1.0	11.2	4.0	1.7	2.4	1.2
	CPD	1.0	8.6	2.3	1.5	1.5	1.1

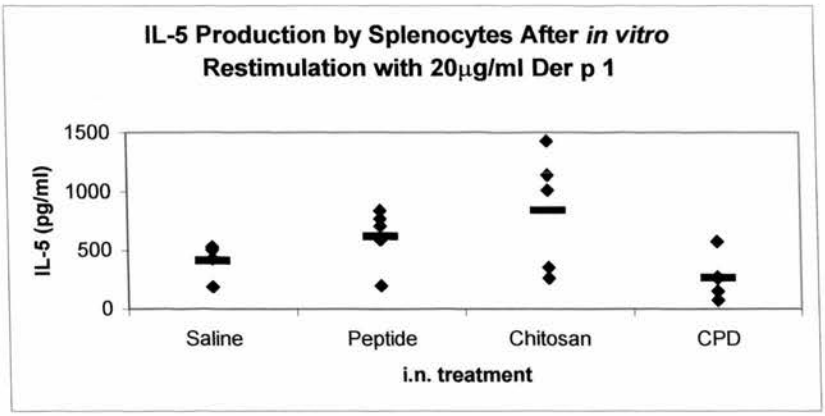
Table 5.1b Cytokine Production by Splenocytes After in vitro Restimulation with 20μg/ml Der p 1

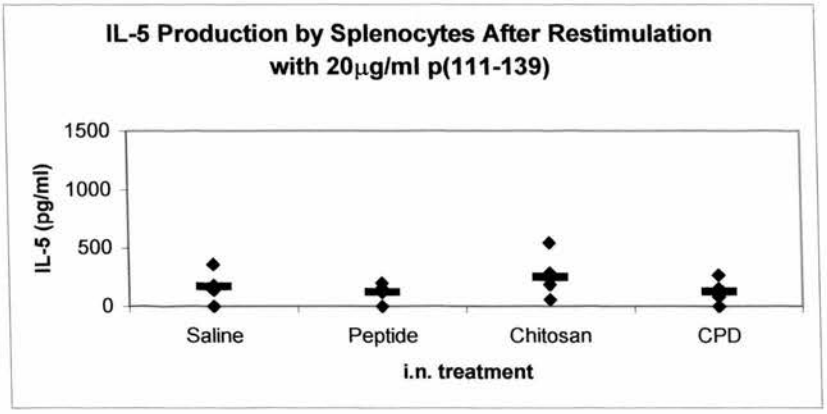
	IL-5	**IL-13**	**IFN-γ**	**IL-10**
Saline	-	-	-	-
Peptide	↑50%	↑103%	↓34%	↑23%
Chitosan	↑103%	↑237%	↓87%	↑35%
CPD	↓35%	↓8%	↓78%	↓31%

The percentages show either an increase or decrease in cytokine production in relation to the saline control.

Table 5.1c Ratio of IL-5:IL-10 production in tracheal lymph node cells after *in vitro* restimulation with Der p 1.

	IL5:IL10
Saline	8
Peptide	8
Chitosan	21
CPD	2





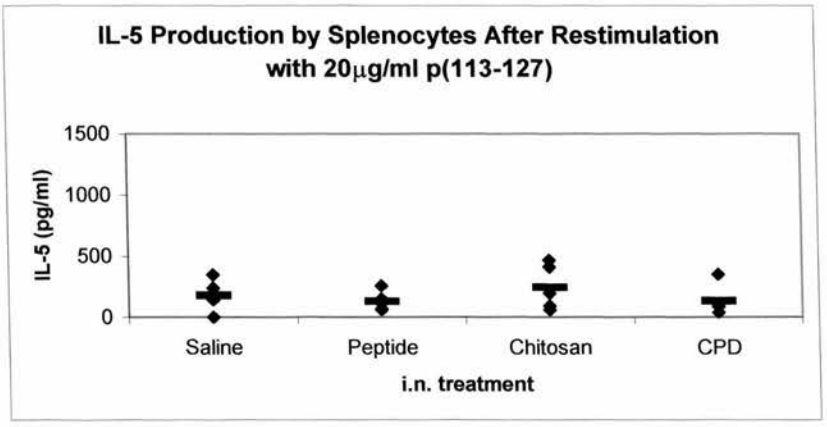
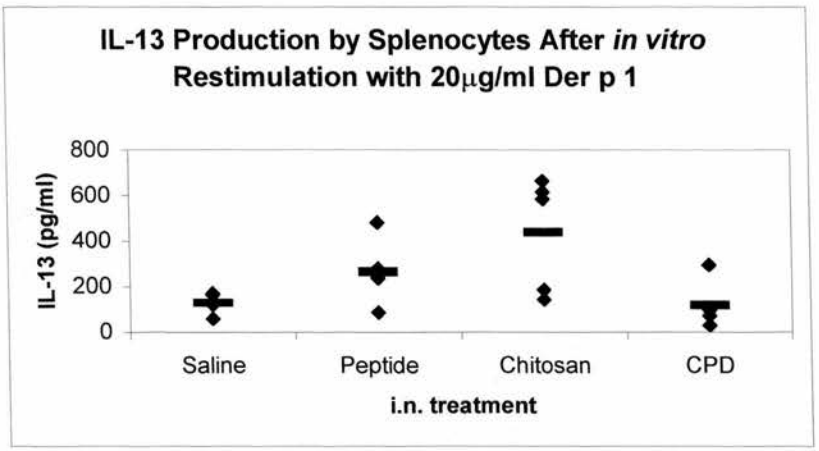
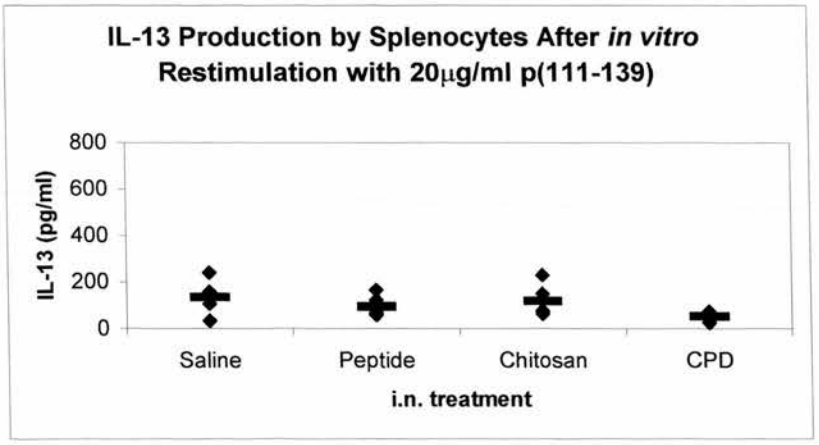


Figure 5.10 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.
IL-5 production by splenocytes.
Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA. Individual mice are represented by diamonds, mean values within each group are represented by bars.
Results for Der p 1;p111-119 and OVA-restimulation are not shown as no detectable IL-5 was produced.





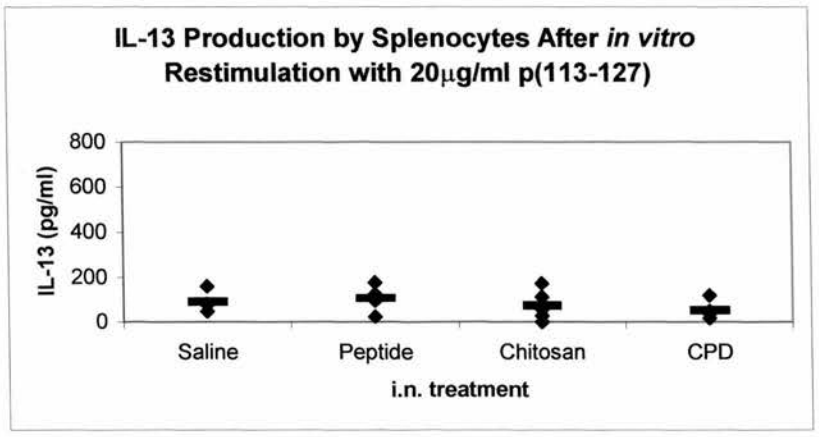


Figure 5.11 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.
IL-13 production by splenocytes.
Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA. Individual mice are represented by diamonds, mean values within each group are represented by bars.

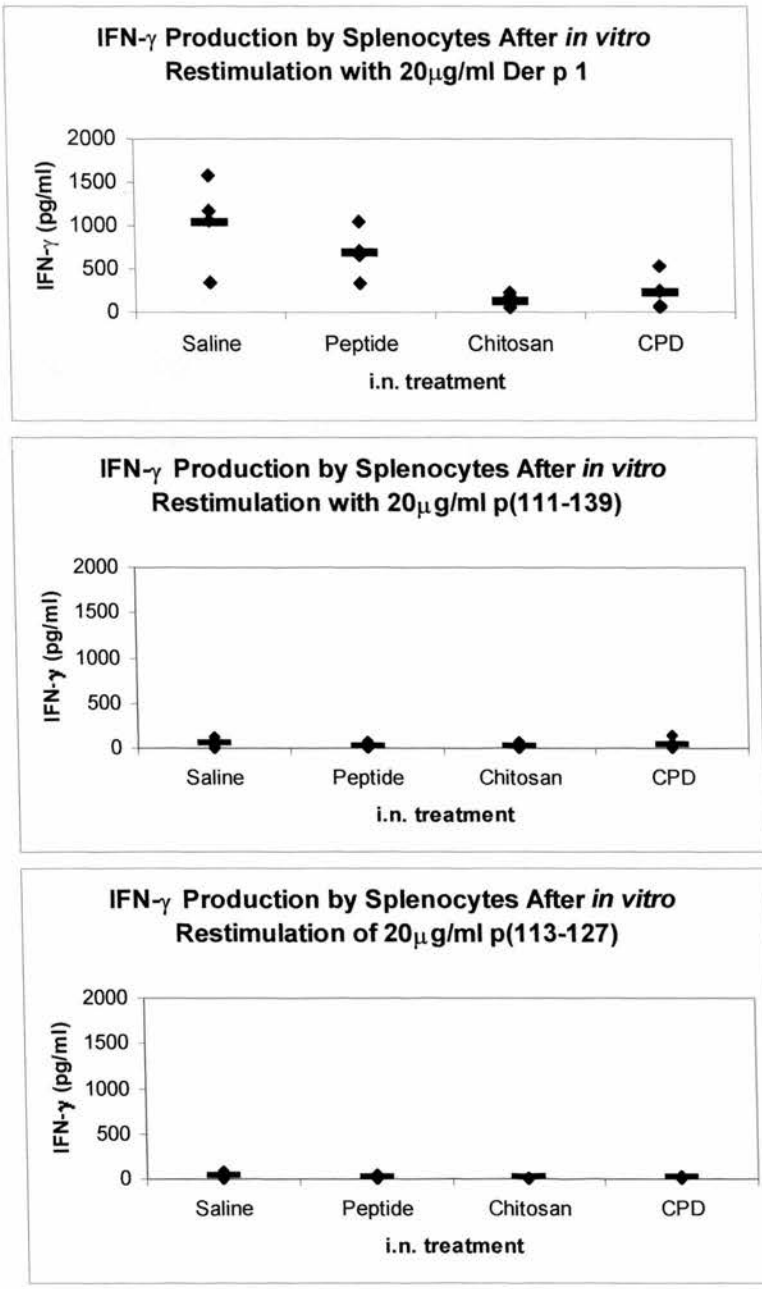
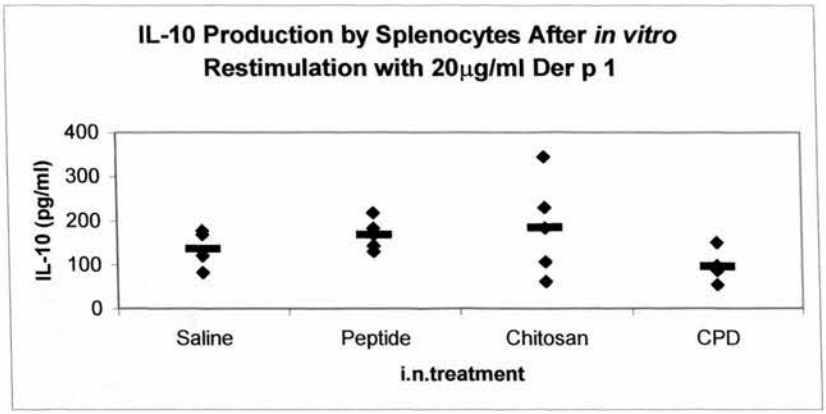
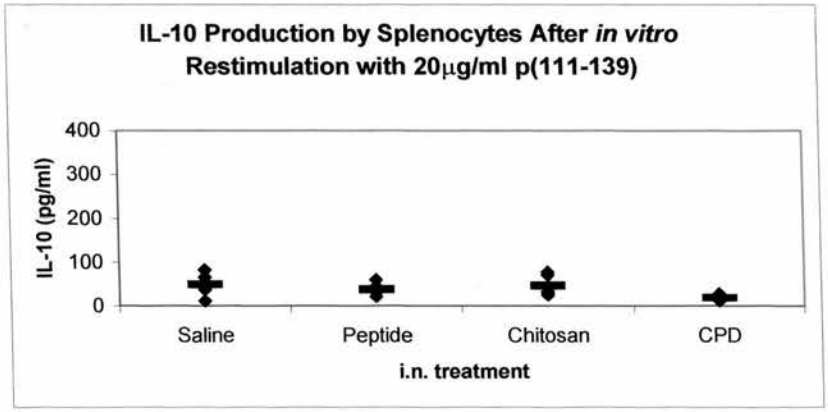


Figure 5.12 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.

IFN-γ production by splenocytes.

Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA. Individual mice are represented by diamonds, mean values within each group are represented by bars.





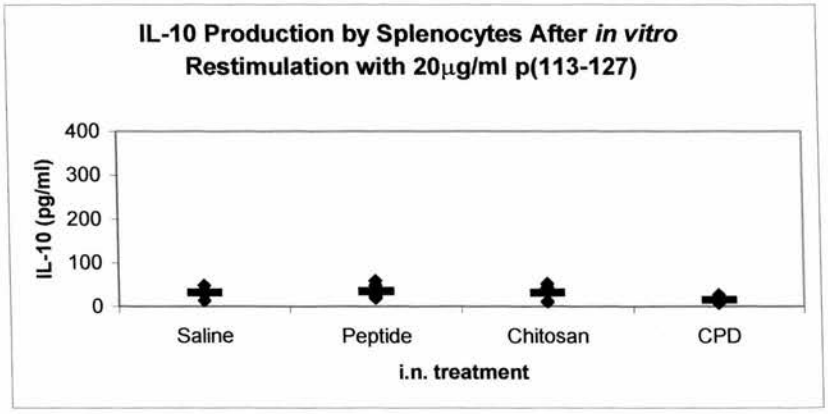
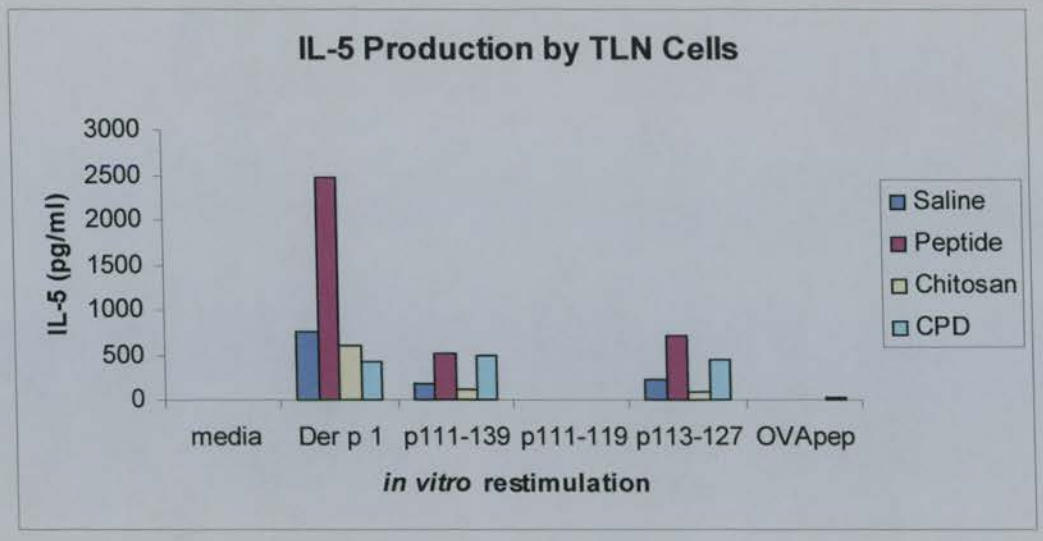


Figure 5.13 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.
IL-10 production by splenocytes.
Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA. Individual mice are represented by diamonds, mean values within each group are represented by bars.



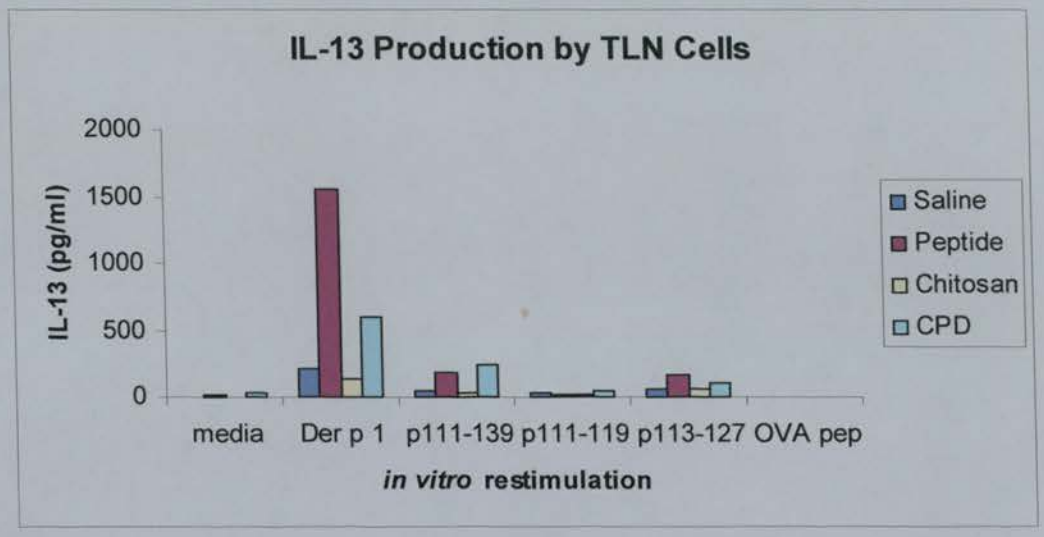
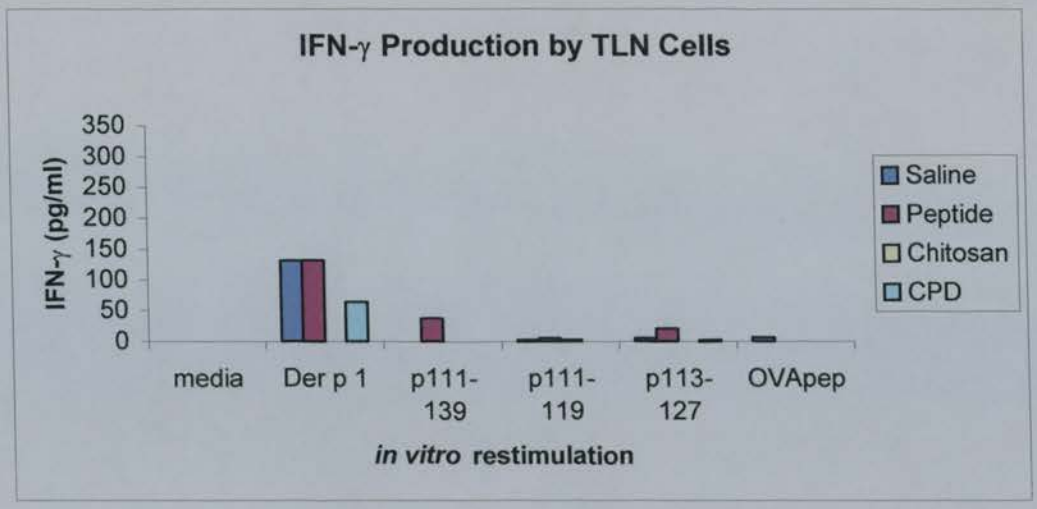


Figure 5.14 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.
IL-5 and IL-13 production by TLN cells.
Single cell suspensions from pooled tracheal lymph nodes (5 x 10^5/well TLN cells) were cultured with media alone or media supplemented with 20 μg/ml Der p 1; p(111-139), p(111-119), p(113-127), OVA peptide and α-CD3/CD28. Supernatants were removed at 72 hours for cytokine determination.
Results for α-CD3/CD28 are not shown.
Mean +/- SE is shown.



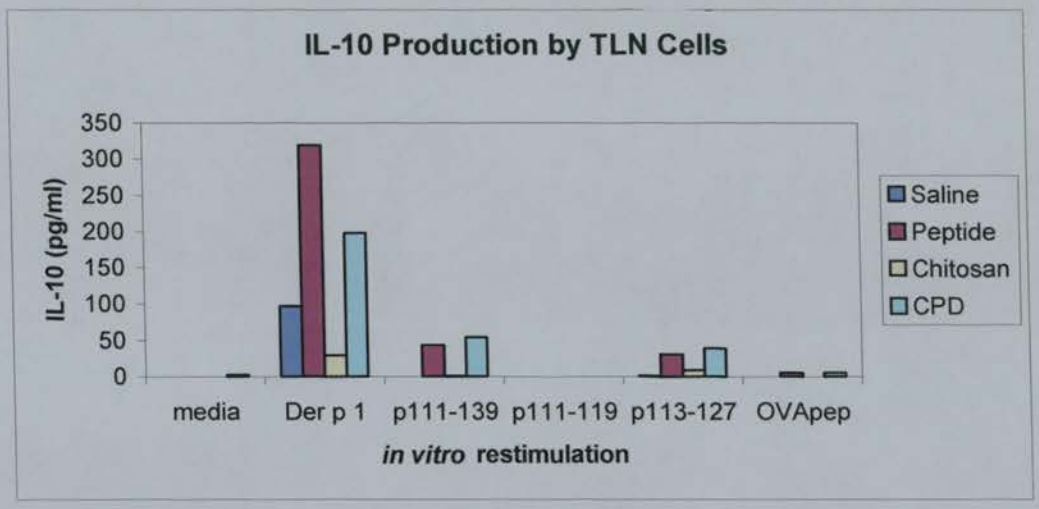


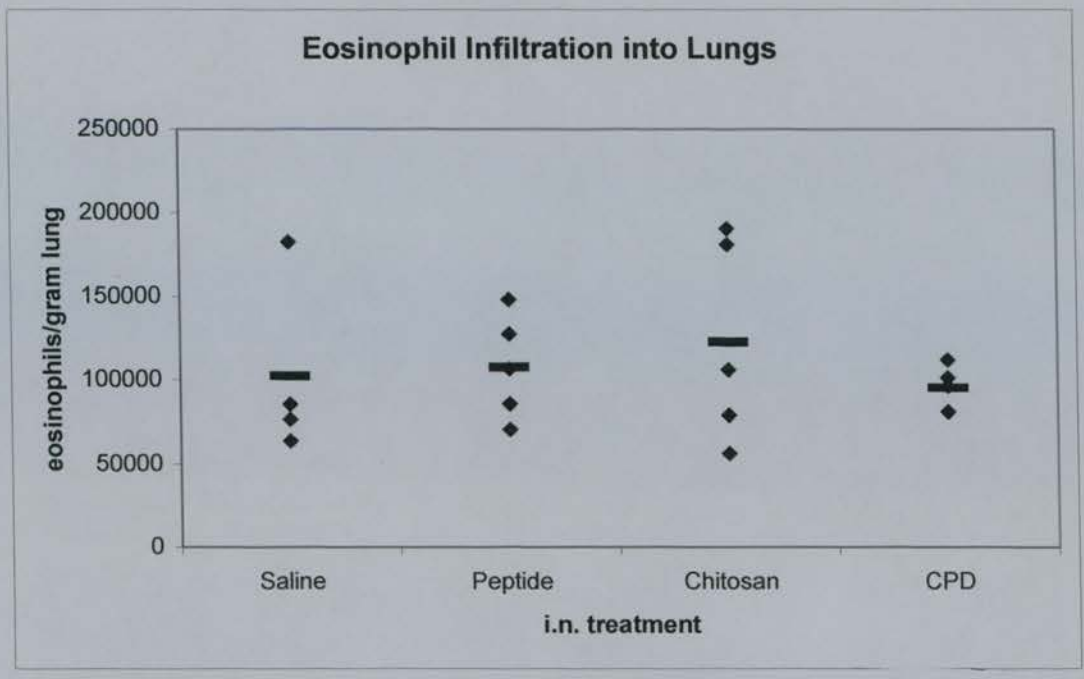
Figure 5.15 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.

IFN-γ and IL-10 production by TLN cells.

Single cell suspensions from pooled tracheal lymph nodes (5 x 10^5/well TLN cells) were cultured with media alone or media supplemented with 20 μg/ml Der p 1; p(111-139), p(111-119), p(113-127), OVA peptide and α-CD3/CD28. Supernatants were removed at 72 hours for cytokine determination.

Results for α-CD3/CD28 are not shown.

Mean +/- SE is shown.



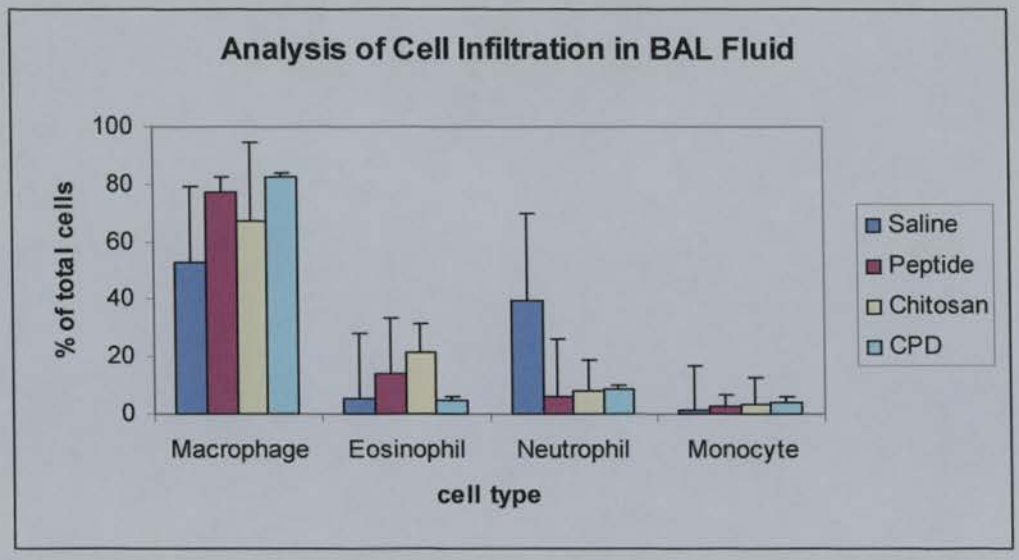
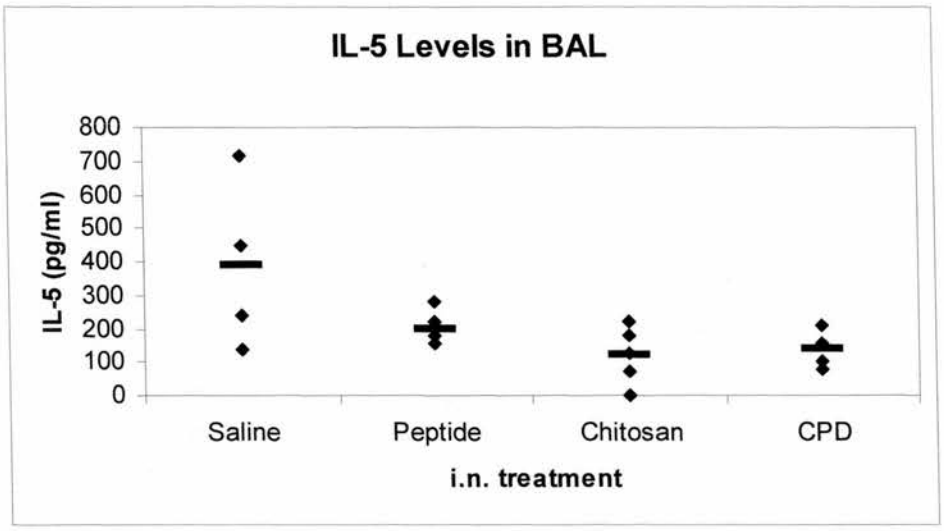


Figure 5.16 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.
Eosinophil infiltration into lungs and BAL fluid.
Lung eosinophil infiltration was detected by an eosinophil peroxidase assay.
The upper chart shows eosinophil infiltration into the lungs levels of individual mice. Individual mice are represented by diamonds, mean values within each group are represented by bars.
The lower chart shows the analysis of cell infiltration into BAL fluid using cytospins. Mean +/- SE is shown.



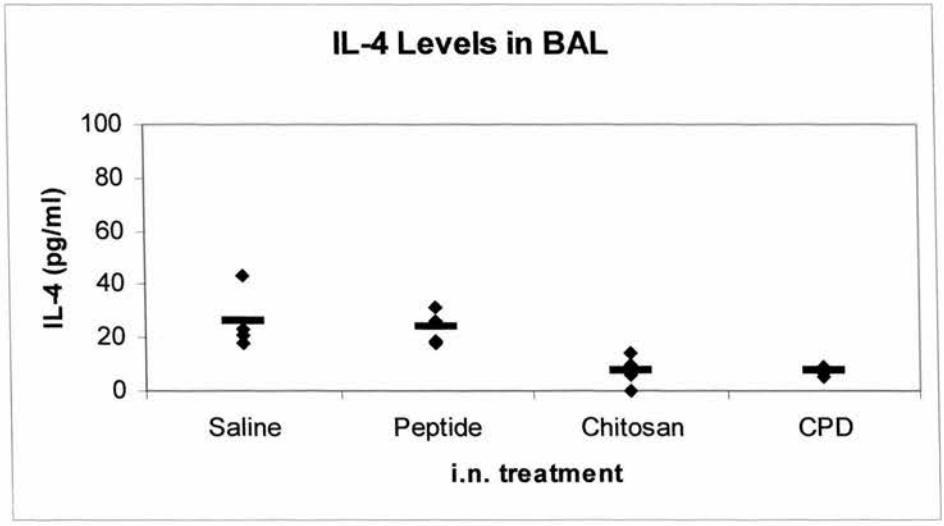


Figure 5.17 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.
IL-5 and IL-4 levels in BAL fluid.
Mouse lungs were lavaged in situ after death with PBS. The lavage was centrifuged to remove the cellular infiltrate (used for cytospins). The supernatant was frozen at -70° until analysis by ELISA. The charts represent individual mice (diamonds) as well as mean values within each experimental group (lines).

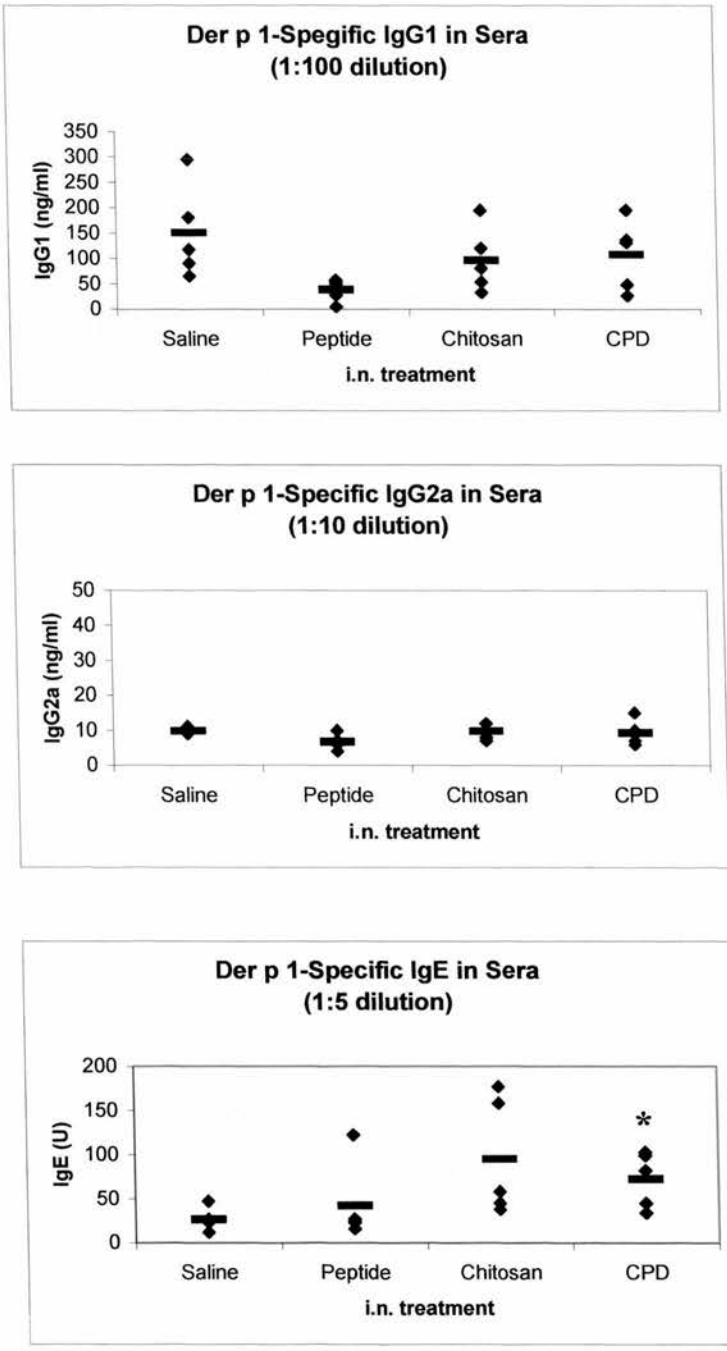


Figure 5.18 - Intranasal administration of peptide adsorbed to chitosan protects against the induction of Th2 mediated responses in a murine model of pulmonary inflammation.
Der p 1-Specific IgG1, IgG2a and IgE levels in sera.
Antibody levels were measured by ELISA. The charts represent individual mice (diamonds) as well as mean values within each experimental group (lines). Significant differences between treatment and control groups are indicated ($P < 0.05$).

5.4 Intranasal instillation of chitosan and Der p 1;p111-139 after systemic Der p 1 sensitisation.

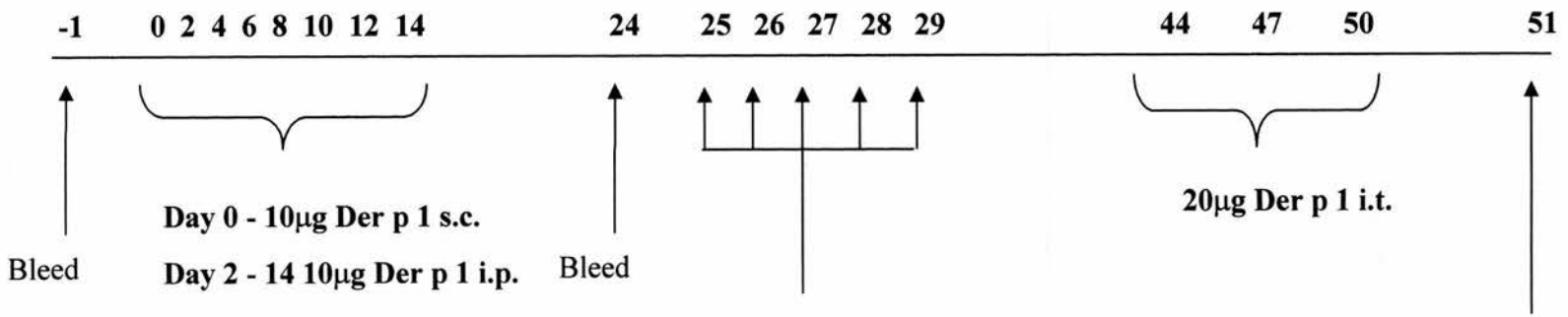
The purpose of this experiment was to investigate whether the intranasal instillation of Der p 1;p111-139 adsorbed to chitosan could down-regulate established Th2 cytokine mediated allergic responses.

The immunisation protocol was chosen because we wanted to investigate a therapeutic (treatment) regimen which involves sensitising mice to Der p 1 using an adjuvant free protocol over the prophylactic regimen where mice are immunised with Der p 1 in alum. The protocol is shown in detail on the following page. Mice were immunised systemically by i.p. injection of 10 μg concentrations of Der p 1 in saline. Eleven days after the immunisation, groups of mice received either saline alone, chitosan alone, Der p 1;p111-139 alone and Der p 1;p111-139 adsorbed to chitosan. The time period between initial sensitisation and 'treatment' was shortened slightly (compared to previous protocols) due to time constraints. After 15 days the mice were challenged by intratracheal instillation on 3 occasions at 3 day intervals. The mice were sacrificed 24 hours after the final intratracheal challenge.

Intranasal instillation of chitosan and Der p 1;p111-139 after systemic Der p 1 sensitisation.

Protocol

Day



a) = saline i.n. (n=7)

b) = 0.25% w/v chitosan i.n. (n=7)

c) = 100μg Der p 1;p111-139 i.n. (n=7)

d) = 100μg Der p 1;111-139 + chitosan i.n. (n=8)

Results

In this study we investigated whether the mucosal delivery of Der p 1;p111-139 to mice primed by systemic immunisation with Der p 1, would protect mice from subsequent development of airway inflammatory responses after allergen challenge; and, if so, whether tolerance was the underlying mechanism.

Clinically, patients are already sensitised to allergens when immunotherapy begins. Thus, it would be more physiological if a protocol was adopted that measured the effects of the administration of a candidate vaccine, in this case Der p 1;p111-139 adsorbed to chitosan after prior systemic sensitisation. The protocol is shown in detail on page 217.

Proliferative responses of allergen reactive T cells

Figure 5.19 shows the proliferation of splenocytes and tracheal lymph node cells after *in vitro* restimulation with various stimuli. Splenocyte proliferation was relatively low (counts < 15,000 cpm) and there was no difference between groups. The most striking observation was the proliferation of tracheal lymph node cells from peptide-treated mice. The intranasal instillation of peptide significantly increased the *in vitro* proliferative response to Der p 1 and related peptides. Counts were at least twice as high as any other group. This suggests that the intranasal administration of Der p1;p111-139 primed localised responses in the draining lymph nodes, leading to enhanced proliferative responses when the airways were later challenged with whole Der p 1. Interestingly, when peptide was co-administered with chitosan, proliferation was reduced to similar levels observed with the saline-treated animals.

Cytokine production by allergen reactive T cells

IL-5 production by splenocyte and tracheal lymph node cells is shown in figure 5.20. IL-5 production by splenocytes was below the threshold of detection of the assay. However, IL-5 was detectable in the tracheal lymph node supernatants. Like the proliferation results, the peptide-treated mice produced the largest amount of IL-5 in response to *in vitro* restimulation with Der p 1 (mean = 7.4 ng/ml +/- 0.4),

Der p 1;p111-139 (mean = 9.4 ng/ml +/- 0.5) and Der p 1;p111-127 (mean = 5.5 ng/ml +/- 2.4). Tracheal lymph node cells from the chitosan-treated mice produced the most IL-5 after Der p 1 *in vitro* restimulation (mean = 3.3 ng/ml +/- 0.4). Neither the saline- or chitosan/peptide mice produced detectable levels of IL-5.

IFN-γ and IL-13 production by splenocytes and tracheal lymph node cells was also tested. Neither of these cytokines were detected in the culture supernatants as the levels were below the threshold of sensitivity of the ELISAs.

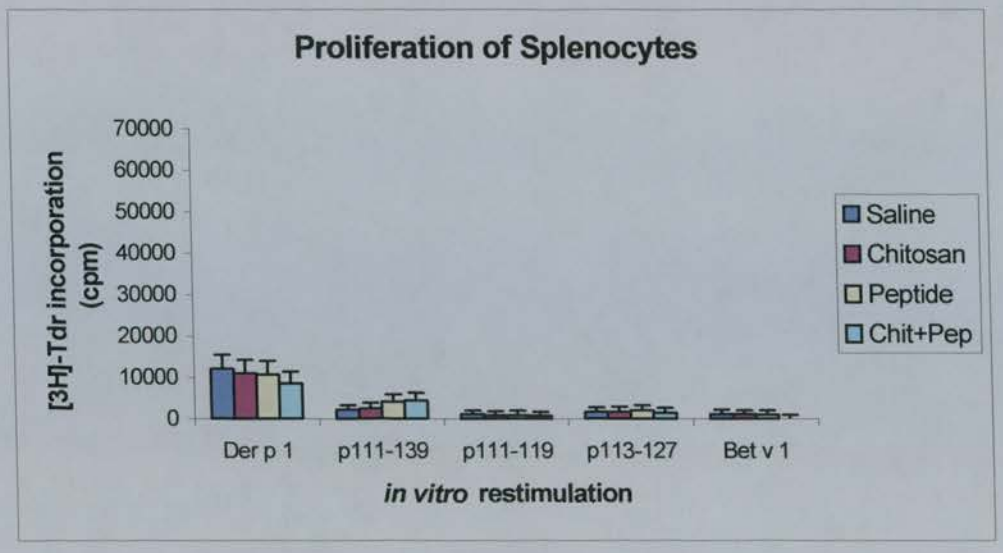
The presence of IL-5, IL-9 and eotaxin in BAL fluid was also investigated. However, none of these cytokines/chemokines were detected.

Effect of intranasal peptide treatment on antigen specific immunoglobulin levels induced following systemic immunisation.

Figure 5.21 shows the levels of Der p 1-specific IgG1, IgG2a and IgE in sera. With each antibody isotype, there was little difference between the groups. IgG1 was the most abundant antibody.

Effect of intranasal peptide administration on eosinophil levels in Der p 1 allergen challenged airways.

Eosinophil infiltration into lungs was also determined and is shown in figure 5.22. There was a large amount of inter-group variation, however the chitosan/peptide treated mice had levels of eosinophilia 43% less than the saline group, with the peptide treated mice only showing a 16% reduction.



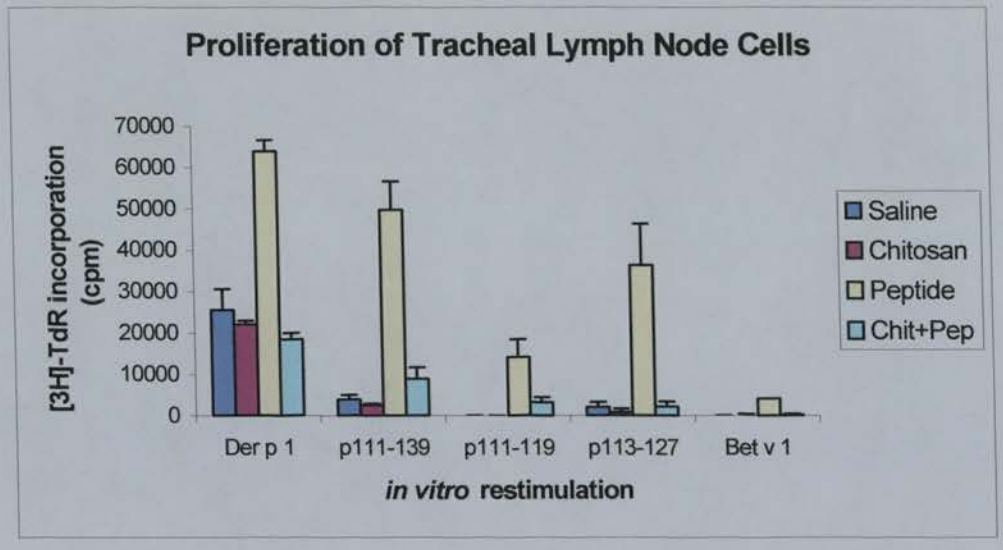
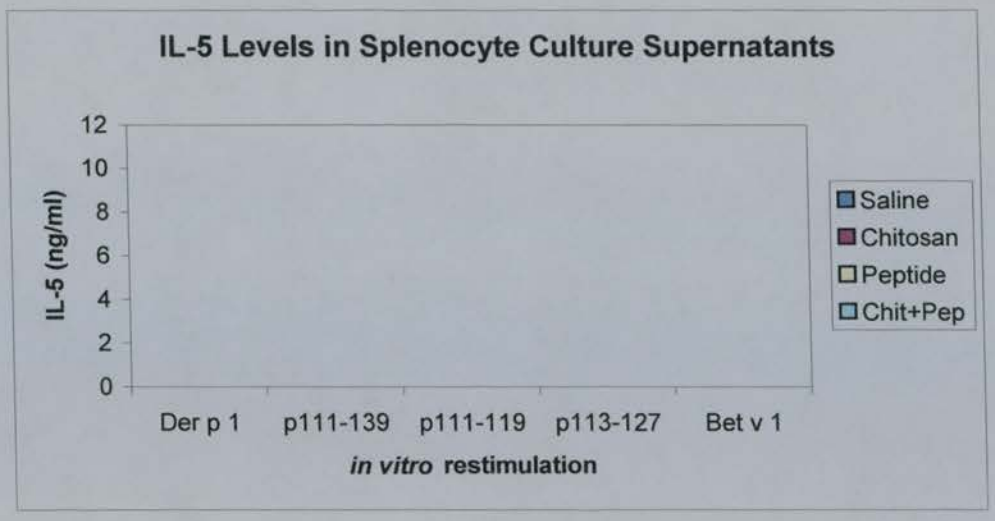


Figure 5.19 – Intranasal administration of chitosan and peptide after prior systemic sensitisation to Der p 1.
Proliferation of splenocytes and tracheal lymph node cells.
C57BL/6 mice were immunised using the protocol outlined on page 217. Single cell suspensions were prepared from pooled tracheal lymph nodes and individual spleens (results pooled), which were then cultured ($5x10^5$/well) with media alone or media supplemented with 10 μg/ml Der p 1, p(111-139), p(111-119), p(111-137) and Bet v 1. A positive control of CD3/CD28 (5 μg/ml/2.5 μg/ml) was also included in the study but results are not shown. Proliferation was measured at 90 hours after pulsing with thymidine at 72 hours.
Mean +/- SE is shown.



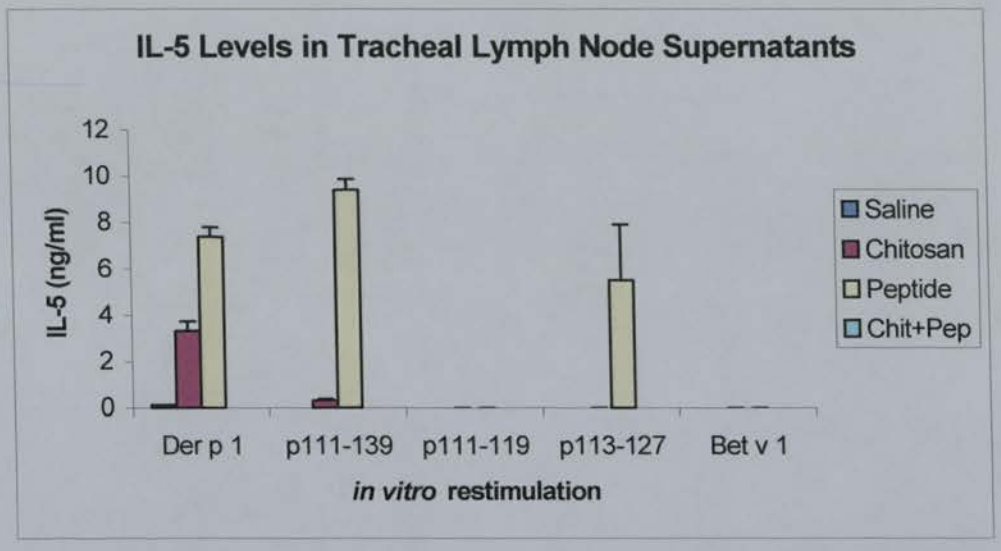
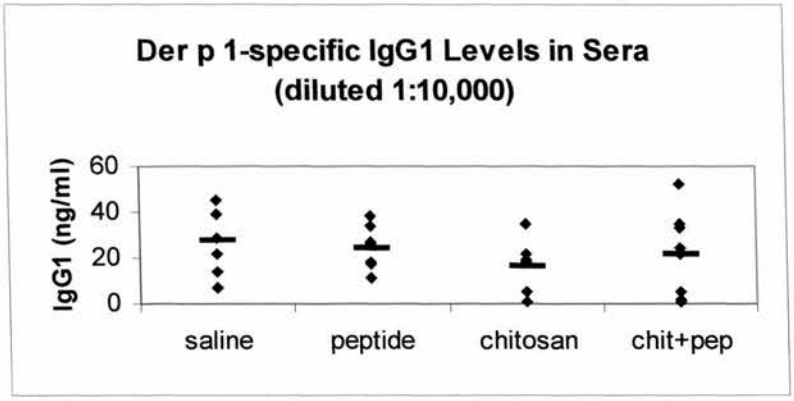
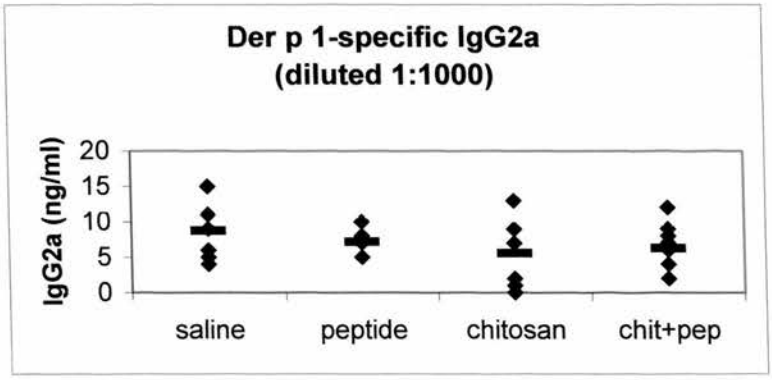


Figure 5.20 - Intranasal administration of chitosan and peptide after prior systemic sensitisation to Der p 1.
IL-5 levels in splenocyte and tracheal lymph node culture supernatants.
The legend describes the intranasal treatment administered to the mice. Single cell suspensions were prepared from pooled tracheal lymph nodes and individual spleens (results pooled), which were then cultured (5 x 10^5/well) with media alone or media supplemented with 10 μg/ml Der p 1, p(111-139), p(111-119), p(111-137) and Bet v 1. Supernatants were removed at 72 hours and frozen at -20°C until analysis by ELISA.
Mean +/- SE is shown.





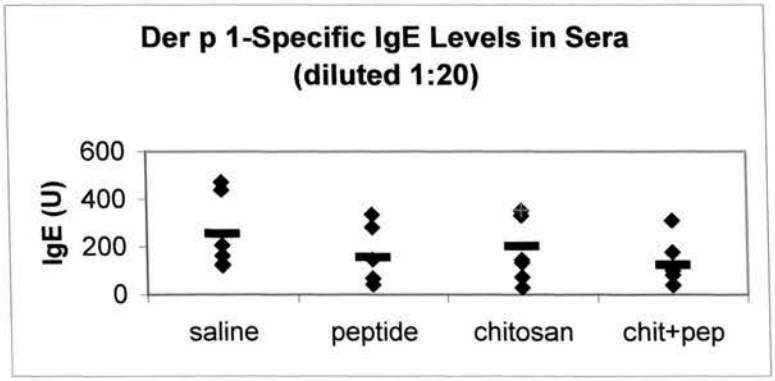
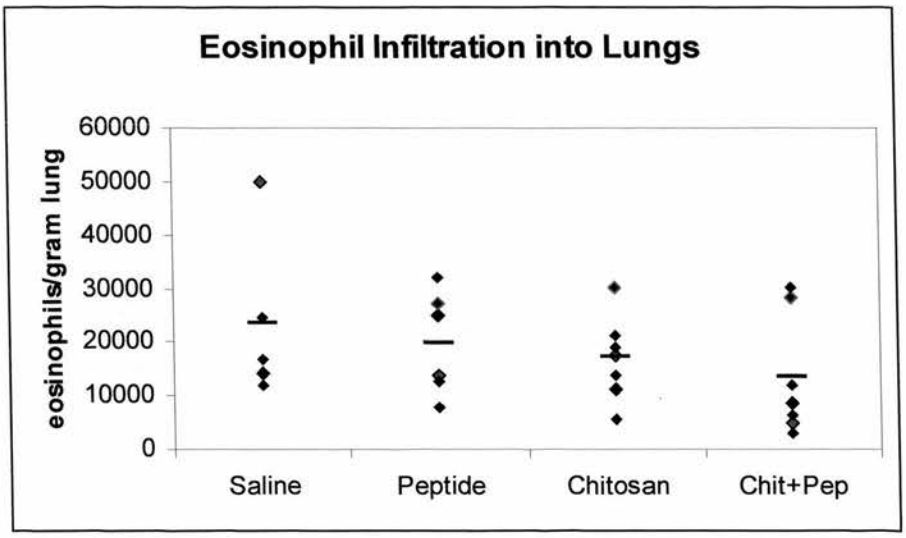


Figure 5.21 - Intranasal administration of chitosan and peptide after prior systemic sensitisation to Der p 1.
Der p 1-specific levels of IgG1, IgG2a and IgE in sera.
The presence of antibodies was detected by ELISA.
Individual mice are represented by diamonds. Mean values are represented by bars.



	Decrease in eosinophilia in relation to the saline control
Saline	-
Peptide	16%
Chitosan	28%
CPD	43%

Fig. 5.22 - Intranasal administration of chitosan and peptide after prior systemic sensitisation to Der p 1.
Eosinophil infiltration into lungs.
Each diamond represents an individual mouse. The bars show the mean value for each group.
The lower table shows the decrease in eosinophil production by the other groups of mice in relation to saline controls.

5.5 Discussion

The primary aim of this study was to establish whether chitosan could prevent the induction of and/or reverse allergic inflammation; and secondly, if so, whether the induction of tolerance or Th1 immunity was the underlying mechanism. An important factor in tolerance induction is the affinity of interactions between the TCRs and their ligands which consequently affects the response generated [538-540]. As discussed in the main introduction to this thesis, *in vitro* studies have shown that high affinity TCR-MHC class II peptide interactions prime Th1 responses whereas low avidity interactions prime Th2 responses [57]. Effector T cell responses are also differentially influenced when mice are immunised with altered peptide ligands [47]. Therefore, the selective priming of Th2 responses to inhalant allergens by pulmonary DCs [541] may be partly attributable to low levels of antigen exposure and consequently lower affinity TCR-MHC class II-peptide interactions. A number of studies suggest that chitosan can be used to enhance immunological responses at the nasal mucosa [529-531]. Thus, the intranasal administration of an immunodominant peptide of Der p 1 – Der p 1;p111-139 adsorbed to chitosan was thought to increase the uptake of antigen across the mucosa and thus increase the affinity of the TCR-MHC class II interactions promoting tolerance induction or the priming of Th1 responses.

A previous study by Hoyne et al showed that intranasal administration of Der p 1;p111-139 peptide protected against Th1 mediated DTH responses [501], however, we have consistently failed to induce Th2 cell tolerance to Der p 1 by the intranasal administration of this peptide. In addition, Ebner et al found that administration of increasing doses of Phl p 1 (a major allergen of timothy grass) induced tolerance to the allergen associated with a shift in the cytokine profile of allergen reactive T cells from a Th2 to a Th1 like response [542]. In this study we found that the intranasal administration of Der p1;p111-139 peptide absorbed to chitosan, lead to the hyperresponsiveness of $CD4^+$ lymphocytes at day 2, as a result of *in vitro* restimulation with Der p 1;p111-139 peptide (figure 5.2). Enhanced proliferation and increases in IFN-γ relative to IL-5 in the chitosan/peptide mice was observed (figures 5.3-5.6), compared with T cells isolated from peptide treated mice. This trend is observed in desensitization therapy which involves the administration of

increasing doses of allergen to allergic patients, ie. there is a shift in the cytokine profile of allergen reactive T cells from a Th2 to a Th1 like response and in the induction of T cell tolerance [165, 166, 318, and 542]. The transient increase in IFN-γ production by hyperreactive T cells was also observed in the chitosan/peptide mice that were subsequently rechallenged via the airways with Der p1 allergen. The increase in IFN-γ has been shown to play a protective role in the initial induction of tolerance in naïve animals to inhalant antigen [146] [543].

Verhoef et al showed that the use of an altered TCR ligand derived from the immunodominant T cell epitope of a group 2 allergen of house dust mite, lead to elevated IFN-γ levels compared to the native peptide under activating conditions but under tolerising conditions, lead to the induction of T cell anergy and lower IL-10 production than the native peptide [544]. It is conceivable that subtle changes in the structure of the Der p 1 peptide, introduced by absorption to chitosan, may alter the affinity of the peptide/MHC class interactions with the TCR.

To establish whether intranasal administration of peptide containing the immunodominant T cell epitope of Der p 1 adsorbed to chitosan, protected against subsequent induction of airway inflammatory responses, eosinophilia was measured in the lungs and BAL fluid, following allergen provocation of the airways. Both assays showed similar results with all groups of mice exhibiting similar levels of eosinophilia in the lungs as well as the BAL fluid (figure 5.16). This may be explained by the higher ratio of IL-5:IL-10 in the tracheal lymph node cells of each group of mice (table 5.1c, page 206). As discussed earlier, IL-5 is directly responsible for recruitment of eosinophil progenitor cells from the bone marrow, their differentiation, maturation and survival in the airways [229, 545]. IL-13 is also an important cytokine in the pathogenesis of asthma. When IL-13 production was blocked in OVA-sensitised mice by the administration of a soluble IL-13α2-IgGFc fusion protein, there was a complete reversal of allergen-induced AHR [201]. In addition, mice with a targeted deletion of IL-13 failed to develop allergen-induced AHR, despite the presence of vigorous Th2-biased, eosinophilic pulmonary inflammation [546]. Therefore, it is possible that the combined effects of IL-5 and IL-13 have overridden the immunoregulatory effects of the IL-10.

The investigation into the cytokine production by the Der p 1 specific T cells in the spleen and the tracheal lymph nodes revealed the existence of a dichotomy. Splenic production of IL-5, IL-13 and IFN-γ was reduced in mice that had received peptide adsorbed to chitosan (figures 5.10-5.12), indicating that protection against allergen induced effector responses, was due to the induction of antigen specific T cell tolerance and not a shift in cytokine production. In contrast, Der p 1 reactive T cells from the tracheal lymph nodes draining the airways, did not show reduced levels of IL-5 and IL-13 production (figure 5.14), nor was there an increase in levels of IFN-γ (figure 5.15). However, there was an antigen dependent increase in IL-10 production (figure 5.15) by cells isolated from the draining tracheal lymph nodes, following allergen challenge. The fact that the production of IL-10 was antigen-dependent, indicated that the Der p 1 reactive T cells were the principal source of this immunoregulatory cytokine in the draining lymph nodes.

Previous studies have demonstrated that the intranasal administration of an immunodominant epitope of myelin basic protein induced IL-10 production by splenic T cells indicating that IL-10 producing regulatory T cells may also play a role in the induction of peripheral tolerance [509]. In addition, Stämpfli and co-workers demonstrated that administration of IL-10 into the lungs, using replication-deficient adenovirus vectors in a murine model of airway hyperreactivity during the time of mucosal sensitization, abrogated both the cellular and physiologic recall responses *in vivo*. The prevention of airway eosinophilia by IL-10 was independent of IFN-γ and was associated with decreased production of IL-4, IL-5, and TNF-α in the lungs [547]. Thus, the evidence suggests that IL-10 is a critical regulatory molecule involved in the prevention of asthma.

It is possible to stimulate the generation of T regulatory (Tr1) cells *in vitro* by stimulating $CD4^+$ T cells with antigen in the presence of IL-10. These Tr1 cells produce significant amounts of IL-10 as well as IFN-γ, TGF-β and IL-5, but not IL-2 and IL-4 [512]. Also, when $CD4^+CD45RB^{low}$ Tr1 cells were co-transferred with pathogenic $CD4^+CD45RB^{high}$ cells into SCID mice, there was protection against inflammatory bowel disease, indicating that these cells were functionally active *in vivo* [337, 512, 548]. Recently it has been demonstrated that *in vitro* generated Tr1 cells can inhibit the priming of Th2 responses *in vivo* following transfer [514].

Pulmonary DCs are thought to play a critical role in the induction of tolerance to inhalant antigen and in the generation of Tr1 cells. A recent study by Akbari et al showed the migration of pulmonary DCs to the tracheal lymph nodes following inhalation of antigen. The DCs expressed a mature phenotype and produced IL-10. Furthermore, pulmonary DCs obtained from intranasal peptide treated wild type but not IL-10 knock out mice, could adoptively transfer tolerance to naïve mice and *in vitro*, prime IL-10 and IL-4 producing $CD4^+$ Tr1 cells [511].

Although a significant reduction in eosinophilia was not observed in chitosan/peptide mice treated prior to systemic challenge with Der p 1 (figure 5.18), there was a reduction in eosinophilia in mice that received chitosan/peptide after systemic immunisation with Der p 1. This data suggests that these antigen specific Tr1 cells may play an important role in maintaining tolerance through production of IL-10 in lymph nodes draining the airways following antigen challenge.

In this study, an inhibition of antibody production following immunisation with the intact Der p 1 antigen was not observed. Mice that received chitosan/peptide showed no reduction in serum IgG levels and IgE production was actually increased. A recent study by Hurst and colleagues found that the establishment of normal IgE tolerance to inhaled OVA Ag could be subverted by strong local Th2 responses but not Th1 responses, to unrelated antigens or pathogens in the lung, and that this prevention was largely the consequence of local IL-4 production immediately before inhalation of OVA [437]. An intranasal KLH challenge to mice sensitised with KLH/alum before primary exposure to OVA by aerosolisation, resulted in the sensitisation, not tolerance of mice to OVA. Likewise, infection of mice with antigens such as *Nippostrongylus brasiliensis* or exposure to *A. fumergatus* extract, both of which produce vigorous Th2 responses, prevented the establishment of IgE tolerance to aerosolised OVA. Therefore, it may be possible that the mice in this chitosan study have been exposed to some infectious agent or pathogen that has enhanced the Th2 environment before the primary intranasal challenge with Der p 1 peptide. In addition, the increase in IgE production may have been due to the effect of chitosan on IFN-γ production, since IFN-γ counterbalances the development of Th2 responses and production of IL-4. The failure to inhibit antibody production, in

spite of significant reductions in Der p 1 reactive T cell effector functions, may be due to the emergence of T cells reactive with minor epitopes of Der p 1.

Finally, it seemed logical to examine the effects of peptide/chitosan treatment on an established allergic response, as in the clinical situation subjects are already sensitised to allergens. Peptide treatment alone greatly enhanced the established Th2 response which was most evident in the tracheal lymph nodes. However, when peptide was co-administered with chitosan, proliferation and IL-5 production were at levels comparable to the saline controls (figure 5.19). Although this is clearly not a reduction in the allergic response below control levels, the comparison with the peptide-treated mice is certainly intriguing. The eosinophil infiltration results suggest that chitosan/peptide treatment may slightly alleviate lung inflammation but the results were not statistically significant (figure 5.22).

As mentioned previously, the absorption of peptide to chitosan could alter its structure thus affecting the affinity of binding to the TCR-MHC II complexes. Also, the bioadhesive properties of chitosan coupled with its ability to form nanoparticles complexes leading to better presentation of antigens to the immune cells of the mucosal epithelial and the nasal associated lymphoid tissue (NALT) may promote the induction of tolerance [536, 549].

To date, this is the first study investigating chitosan as a vehicle for peptide delivery. Many questions remain to be answered but chitosan may have therapeutic potential as a vaccine strategy for preventing the development of allergic asthma in atopic individuals.

Conclusions

This thesis addresses a number of hypotheses. Firstly, we have shown that it is possible to induce an allergen-specific Th2 response with accompanying lung inflammation by challenging the airways with allergen after previous systemic sensitisation. Traditionally, Th2 induction in mice has involved the use of alum adjuvant to drive strong T and B cell-mediated responses, however, in this study, we were able to induce allergen-specific responses of a similar magnitude without the use of adjuvant, thus creating a more physiological system.

However, the main aim of this thesis was to investigate different approaches for the modulation of Th2 immunity to the major house dust mite allergen Der p 1. This was targeted in two ways: either by the induction of antigen-specific tolerance or inhibiting the synthesis of Th2 cytokine production by inducing Th1 immunity.

The administration of microencapsulated Der p 1 and the immunodominant epitope Der p 1;p111-139 peptide was investigated. Microencapsulated Der p 1 failed to induce tolerance or a shift in cytokine production to that of the Th1/Th0 phenotype. However, the intranasal administration of microencapsulated Der p 1;p111-139 suppressed both systemic and local inflammatory responses to intact Der p 1. This was shown by a reduction in IL-4, IL-5 and IL-13 accompanied with a reduction in eosinophilia. The exact mechanism of Th2 suppression was not determined although it was hypothesised that IL-10 played a part. However, further studies would need to be undertaken in order to determine whether IL-10 played a critical role in suppressing Th2 responses.

Furthermore, we showed that IL-10 played a critical role in suppressing Th2 responses, including production of the cytokines IL-4, IL-5 and IL-13 which are associated with the pathology of asthma.

The effects of administering Der p 1;p111-139 peptide with chitosan, an enhancer of epithelial permeability was also investigated. By using a murine model of pulmonary allergic inflammation, we demonstrated that the mucosal delivery of Der p 1;p111-139 could protect against the induction of Th2-mediated airway inflammation and eosinophilia, in mice challenged with whole Der p 1 allergen. The suppression of airway inflammation by intranasal administration of peptide antigen was found to be initiated by T cell hyperactivation and maintained by the production of IL-10 antigen-specific T cells in the draining lymph nodes.

In conclusion, we have described two possible methods of regulating the immune response in a murine model of allergic disease. Further work needs to be undertaken to elucidate the exact mechanisms involved in this immunoregulation but these studies provide exciting new evidence of possible immune therapies that may be utilised in the future.

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Appendix

Kinetics and mode of peptide delivery via the respiratory mucosa determine the outcome of activation versus T_H2 immunity in allergic inflammation of the airways

Gillian Hall, BSc,[a] Lise Lund, PhD,[b] Jonathan R. Lamb, PhD,[a] and Elizabeth R. Jarman, PhD[a,b] *Edinburgh, United Kingdom, and Hørsholm, Denmark*

Background: Specific immunotherapy involving systemic injection of allergen, though highly effective, can cause severe side effects due to IgE-mediated activation of effector cells. Allergen-derived peptides might provide a safer alternative. We have investigated the use of mucosally delivered peptide to induce CD4+ T_H2 cell tolerance and thus protect against allergen-induced airway inflammation.

Objective: The purpose of this study was to investigate whether intranasal administration of an allergen-derived peptide, either alone or adsorbed to chitosan, can prevent the induction of T_H2-mediated pulmonary inflammation after sensitization and challenge of the airways with allergen.

Methods: Mice were given (intranasally) a peptide containing an immunodominant epitope of the *Dermatophagoides pteronyssinus* (Der p) 1 allergen, either as soluble antigen or adsorbed to chitosan, before sensitization and allergen challenge. Pulmonary inflammation, antigen-specific CD4+ T-cell responses, and antibody levels in sera were then determined.

Results: Mice given peptide adsorbed to chitosan had significant reductions in airway eosinophilia, which correlated with reduced levels of IL-4 and IL-5 in the bronchoalveolar lavage fluid. There was decreased recruitment of activated CD4+ T cells into the airways after allergen challenge, which correlated with a loss of Der p 1–specific T-cell cytokine responses in the periphery and the localized production of IL-10 by antigen-specific T cells in bronchial lymph nodes. Induction of peripheral T-cell tolerance was preceded by transient T-cell activation and IFN-γ production.

Conclusion: Our data demonstrate that suppression of airway inflammation by intranasal administration of peptide antigen adsorbed to chitosan is initiated by transient T-cell activation and maintained by the production of IL-10 by antigen-specific T cells in the draining lymph nodes. (J Allergy Clin Immunol 2002;110:883-90.)

Key words: *Allergy, tolerance, immunotherapy, in vivo animal models*

Allergic asthma is characterized by elevated serum IgE levels, airway hyperresponsiveness, and bronchial inflammation after exposure of the airways to inhaled allergens. CD4+ T_H2 cells expressing an activated memory phenotype and the cytokines IL-4, IL-5, IL-9, and IL-13 have been isolated from the bronchoalveolar lavage fluid (BALF) of acute allergic asthmatic individuals and are associated with the disease pathology.[1,2]

The type of effector T-cell response generated in vivo is determined by antigen concentration, route of delivery, and the presence of inflammatory signals, which influence antigen-presenting cell (APC) activation, upregulation of costimulatory molecules, and cytokine production. Inflammatory signals are required for maturation of dendritic cells (DCs) in the airway mucosa, upregulation of MHC class II proteins, the costimulatory molecules (CD80 and CD86), and chemokine receptor CCR7, which is critical for migration of DCs to the draining lymph nodes.[3,4] The interaction between APCs and CD4+ T cells during antigen presentation and the cytokine milieu influence the primary response generated.[5] CD4+ T-cell activation involves ligation of the specific T-cell receptor (TCR) by MHC class II peptide complexes. In the absence of APC-derived costimulatory signals, TCR ligation leads to functional inactivation and anergy.[6] As in activation, a critical threshold level of signaling needs to be reached for induction of T-cell tolerance, which is preceded by and critically dependent on an initial state of transient activation, upregulation of TCR and CD69 expression, and increased proliferative and cytokine responses, after antigen-specific TCR ligation.[7] Peripheral T-cell tolerance can be induced by the mucosal delivery of soluble antigen.[8] Studies in animal models have demonstrated that intranasal administration of allergens to the upper respiratory tract before parenteral immunization can suppress the induction of peripheral allergen-specific lymphoproliferative T-cell responses and IgE production.[9-11] The effect on T cell–mediated inflammatory responses of the lower airways requires further investigation. Clinical studies have demonstrated that subcutaneous immunotherapy using peptides can induce peripheral T-cell tolerance with attenuation of allergen-induced late-phase cutaneous, but

From [a]the Immunobiology Group, MRC Centre for Inflammation Research, Respiratory Medicine Unit, University of Edinburgh Medical School, and [b]ALK-Abello A/S, Hørsholm.

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Correspondence and reprint requests: E. R. Jarman, PhD, ALK-Abello A/S, Boege Alle 6-8, DK-2970 Hørsholm, Denmark.

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Abbreviations used
APC: Antigen-presenting cell
BALF: Bronchoalveolar lavage fluid
DC: Dendritic cell
Der p 1: Group 1 allergen derived from *Dermatophagoides pteronyssinus* species
pDC: Pulmonary dendritic cell
TCR: T-cell receptor

not asthmatic, reactions.[12] In this study, we investigated whether intranasal administration of a peptide encoding an immunodominant T-cell epitope of Der p 1,[13] a major aeroallergen of house dust mite, can suppress induction of $CD4^+$ T-cell responses and airway eosinophilia after allergen challenge in a murine model of pulmonary inflammation.

Peptide absorption across the nasal mucosa occurs principally by passive diffusion across intercellular barriers formed by tight junctions. To reach a critical TCR activation threshold for tolerance induction, we administered Der p 1; p111-139, the immunodominant T-cell epitope of Der p 1, as a soluble peptide adsorbed to chitosan. Chitosan is a nontoxic, biocompatible, and biodegradable mucopolysaccharide that enhances the level of absorption of compounds delivered via the nasal mucosa.[14,15] This is due in part to the bioadhesive properties of chitosan but is also due to the transient opening of the tight junctions between epithelial cells, which form a barrier to the external environment. Because this increase in absorption occurs in the absence of tissue damage or inflammation, the delivery of peptide adsorbed to chitosan could facilitate tolerance induction by increasing the level of peptide released systemically.[16]

METHODS

Animals

C57BL/6 mice, 6 to 8 weeks of age, were used. Animals were purchased from Harlan. Animal care and experimental procedures were conducted in accord with the animal ethics regulations of the Home Office in the United Kingdom.

Antigens

The house dust mite allergen Der p 1 was affinity-purified from spent mite medium through use of the monoclonal antibody 4C1 (Indoor Biotechnologies Ltd). The synthetic peptide Der p 1 p111-139 (FGISNYCQIYPPNANKIRELAQPQRYCR) was synthesized with a free carboxyl terminus and HPLC-purified (Advanced Biotechnology Centre, Imperial College School of Medicine, Charing Cross Hospital, London). The chitosan–Der p 1 p111-139 peptide solution was made up as follows: 2% chitosan in acetic acid (0.5% w/v; Vanson Inc) was dissolved by sonication, diluted further in d H_20 (0.4% w/v), and filter-sterilized. The chitosan was used at a final concentration of 0.25% w/v adsorbed to a minimum of 5 mg/mL peptide.

Immunization and tolerance induction

The following optimized treatment regimes were used: Mice were lightly anesthetized under ether, and groups of mice were treated by the intranasal administration, on each of 5 consecutive days, of a 20-μL volume of endotoxin-free saline solution containing 100 μg of either Der p 1 p111-139 peptide or chitosan adsorbed to Der p 1 p111-139 peptide. The control groups received either saline solution or chitosan alone. Mice were immunized 14 and 16 days after the last intranasal treatment by the intraperitoneal injection of Der p 1 (10 μg) in saline solution absorbed to an equal volume of aluminum hydroxide adjuvant (Imject Alum; Pierce). On days 41 and 43, mice were challenged by intratracheal instillation of Der p 1 (20 μg) into the airways in 50 μL saline solution and killed after 24 hours by means of a lethal dose of Avertin. To determine antibody levels in sera, mice were bled from the tail vein before being killed. Blood samples were centrifuged at 2000*g*; sera were collected and stored at –70°C. For the mechanistic studies, mice were treated intranasally for 3 consecutive days with saline solution, peptide, or peptide adsorbed to chitosan; after either 48 or 96 hours, the mice were killed and their cervical lymph nodes were removed.

T-cell proliferative responses

We prepared single-cell suspensions from the spleen or lymph nodes by teasing the tissue apart and passing it through a fine wire mesh to remove cell debris after red blood cell lysis. Whole cell populations were cultured in RPMI 1640 medium (Gibco) supplemented with 5% FCS, 50 μmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, and 10 U penicillin/streptomycin (Gibco). To measure specific T-cell proliferative responses, cells (1×10^5/well) were cultured in round-bottomed, 96-well microtiter plates (Costar, Corning Incorporated) in medium with Der p 1 p111-139 peptide (5, 20 μg/mL) or Der p 1 (10 μg/mL). To control for T-cell viability, cells were polyclonally stimulated by being cultured in wells containing immobilized anti-CD3 (145-2C11) and anti-CD28 (37.51; 5 μg/mL). After 72 hours, cells were pulsed with 3H-thymidine (1 μCi/well) and the levels were incorporated after a further 16 hours of culture, as determined by liquid scintillation counting.

Cytokine production

Single-cell suspensions prepared from spleen or lymph nodes were cultured (5×10^6/mL) in complete media containing either Der p 1 or Der p 1 p111-139 peptide (10 μg/mL). Control wells contained (a) medium alone or (b) an irrelevant antigen;OVA (10 μg/mL), or wells were coated with anti-CD3 and anti-CD28 (5 μg/mL) antibodies. After 72 hours supernatants were collected; they were stored at –70°C and later assayed in duplicate for the presence of the cytokines IL-4, IL-5, IL-10, IL-13, and IFN-γ through use of ELISA. Antibodies were purchased and used according to the instructions of the manufacturer (IL-4, IL-10, and IL-13, R& D Systems; IL-5 and IFN-γ, Pharmingen).

Measurement of eosinophil and cytokine levels in BALF

Immediately after sacrifice, the trachea were exposed and cannulated with 27-gauge silicon tubing attached to a needle and syringe. The lungs were instilled through the trachea with 500 μL cold PBS, and the BALF was withdrawn. The volume of BALF collected was measured for each sample. To determine BALF cell numbers and composition, samples were centrifuged (700*g*, 5 minutes, 4°C) and cells were washed (PBS/10% FCS), counted, and resuspended at 1×10^6/mL. BALF (1×10^5) cells in 100 μL were cytospun onto poly(L-lysine)-coated slides (3 minutes at 300 rpm by means of a cytocentrifuge). The slides were air-dried, fixed in methanol, and stained through use of Diff-Quik (Dade-Behring). To determine cytokine levels in BALF, samples were stored at –70°C. Cytokines were analyzed through use of ELISA.

Determination of Der p 1–specific antibody levels in sera

Antibody levels in sera were determined through use of ELISA. Unless otherwise indicated, serum samples and antibodies were diluted in PBS/1% BSA and wells were washed between incubation steps with PBS/0.05% Tween-20. Round-bottomed, 96-well microtiter plates (Costar) were coated overnight at 4°C either with rat antimouse IgE (R35-72; Pharmingen) for detection of specific IgE levels or with Der p 1 (5 μg/mL) diluted in carbonate buffer (0.1 mmol/L NaHCO3, pH 8.2) for detection of specific IgG1 and IgG2a. After washing, nonspecific binding was blocked by incubating with PBS/3% BSA for 2 hours at 37°C. Plates were washed, and serial dilutions of serum samples were added in duplicates (IgE, 1:5, 1:10, and 1:20; IgG1, 1:50, 1:100, and 1:500; IgG2a, 1:10, 1:20, and 1:50). The plates were incubated overnight at 4°C. After washing, biotinylated rat antimouse IgG1 (LO-MG1-2; Serotec) or rat antimouse IgG2a (Lo-MG2a-7; Serotec) was added at 1000 ng/mL; plates were then incubated for 2 hours at room temperature. To determine Der p 1–specific IgE levels in sera, Der p 1 (10 μg/mL) was added and plates were incubated at 37°C for 60 minutes. After washing, plates were incubated for an additional 45 minutes with biotinylated anti–Der p 1 mAb 4C1 (1:1000 dilution). Bound biotinylated antibodies were detected by being incubated with streptavidin-peroxidase for 45 minutes at room temperature and visualized through use of the TMB substrate. Plates were read in a microtiter reader at 450 nm through use of the MRX microplate reader (Dynatech) and Revelation software.

Statistical analysis

The data represent mean values ± SEMs. There were 5 mice in each group. Differences between mean values were analyzed by 1-way ANOVA through use of either the Dunnett multiple comparison parametric test or the 2-tailed Student *t* test (antibody levels) with InStat (GraphPad Software). *P* values less than or equal to .05 were considered statistically significant.

RESULTS

Cytokine production by allergen-reactive T cells isolated from mice treated prophylactically by intranasal administration of peptide before sensitization and challenge with allergen

We investigated whether intranasal administration of peptide leads to the induction of tolerance and a loss of cytokine production or a T_H1/T_H0 shift in the phenotype of the effector T-cell populations. For this purpose, cells isolated from the spleens (Fig 1, *A*) or lymph nodes draining the airways (Fig 1, *B*) of Der p 1–sensitized and –challenged mice were restimulated in vitro with Der p 1; levels of IL-5, IL-13, IL-10, and IFN-γ in culture supernatants were then determined through use of ELISA. Investigation of systemic responses revealed reductions in both T_H2 (IL-5 [65%] and IL-13 [62%]) and T_H1 (IFN-γ [75%]) cytokines in peptide-chitosan–treated mice compared with control mice, suggesting that Der p1–reactive T cells in the spleen were tolerized (Fig 1, *A*). Systemic cytokine responses were not suppressed in mice treated with peptide alone. Furthermore, these mice exhibited marked increases in levels of the T_H2 cytokines IL-5 (3-fold) and IL-13 (7-fold) on stimulation of cells obtained from draining lymph nodes with Der p 1. The differential effect of peptide and peptide adsorbed to chitosan on recall responses in lymph node cultures suggest that the kinetics of mucosal delivery might influence the extent to which naive T cells are primed at sites of antigen exposure.

There is increasing evidence that tolerance induced by mucosal administration of soluble antigen is maintained by antigen-specific T regulatory cells through the production of immunosuppressive cytokines. Increases in IL-10 production in lymph node cultures were observed on restimulation with Der p 1 in mice treated with peptide (70%) or peptide adsorbed to chitosan (51%) compared with control mice. IL-10 production was antigen-dependent. Nevertheless, reductions in T_H2-mediated eosinophilia on rechallenge with antigen were associated with increases in the ratio of IL-10 to IL-5 produced (Fig 1, *C*) rather than with increases in IL-10 alone or increases in the ratio of IFN-γ to IL-5 (data not shown), suggesting that in the context of a T_H2-driven response, mechanisms influencing the initial state of T-cell tolerance induction might play a role in maintaining a state of antigen-specific nonresponsiveness. IL-10 levels produced by cells isolated from the spleen did not differ markedly between groups, suggesting that IL-10 might play a greater role in maintaining tolerance at sites of T-cell priming.

Effect of intranasal peptide treatment on antigen-specific immunoglobulin levels induced after systemic immunization

Because the induction of IgE and IgG1 synthesis by B cells requires a cognate interaction with antigen-specific $CD4^+$ T_H2 cells, we investigated whether the tolerization of T cells reactive with an immunodominant region of Der p 1 by intranasal administration of Der p 1; p111-139 peptide was sufficient to inhibit antibody production after immunization with the intact Der p 1 allergen. Mice given peptide intranasally showed minor reductions in Der p 1–reactive IgG1 and IgG2a levels in sera compared with control mice given PBS. IgE levels, however, were not reduced (Fig 2). Mice given peptide-chitosan showed no reductions in serum IgG levels, though IgE production was increased ($P < .05$). Comparable increases in IgE production were observed in mice given chitosan alone. These increases might reflect the inhibitory effect of chitosan on IFN-γ production (Fig 1, *A* and *B*) and consequently an upregulation of IL-4–dependent humoral responses. This suggests that chitosan might not be the most appropriate vehicle for allergen therapy.

Effect of intranasal peptide administration on eosinophil levels in Der p 1 allergen-challenged airways

The infiltration of eosinophils into the airways after allergen challenge is a major pathologic feature of allergic asthma. To determine whether the intranasal delivery

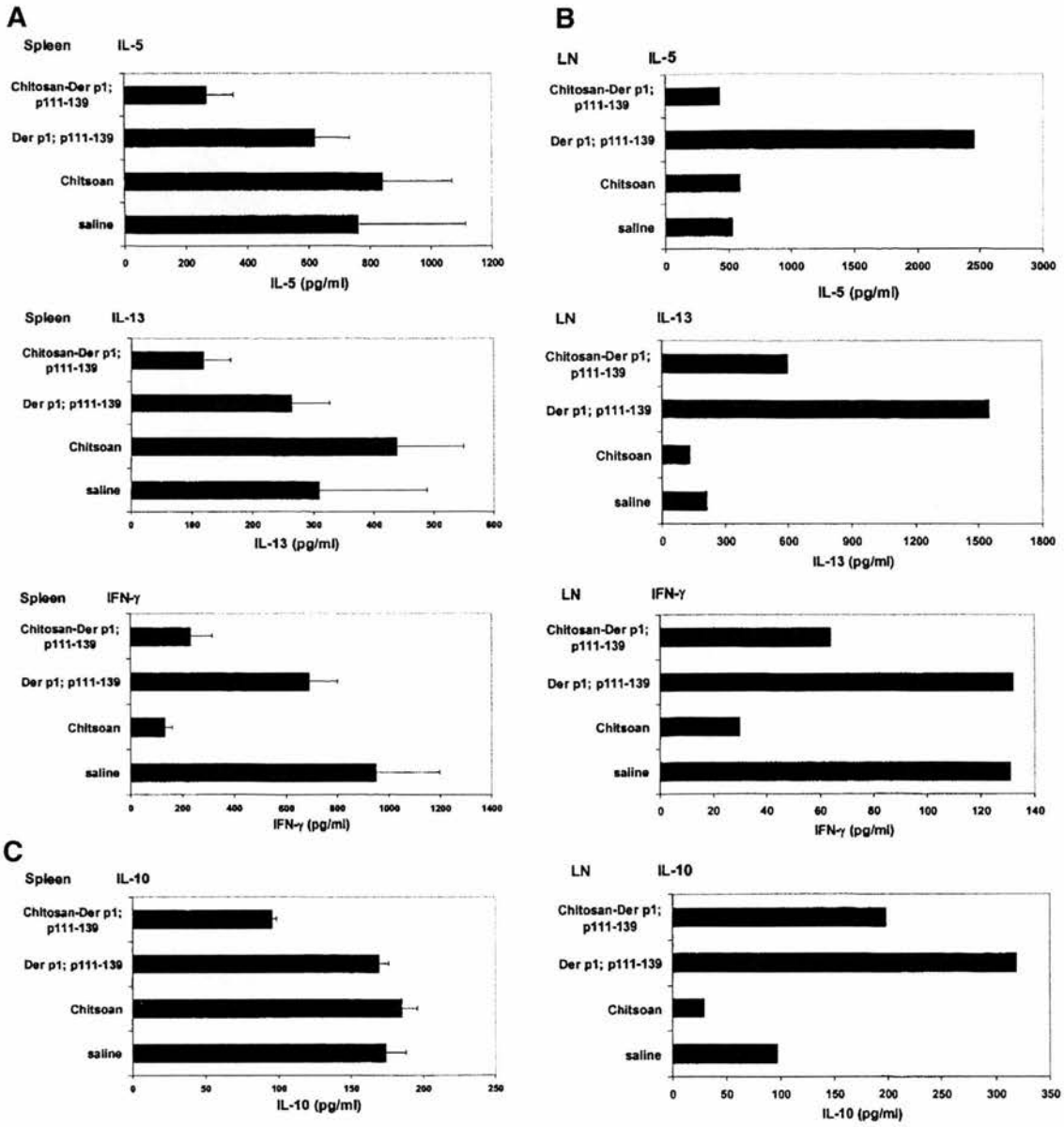

FIG 1. The induction of peripheral T-cell tolerance by the mucosal exposure to antigen is associated with a reduction in both T_H1 and T_H2 responses but also with an increase in IL-10 production. Mice tolerized by the intranasal instillation of peptide alone or peptide adsorbed to chitosan were sensitized and challenged with Der p 1. After 24 hours, cells isolated from spleen **(A)** or pooled bronchial lymph nodes **(B)** were restimulated in vitro with Der p 1 (10 μg/mL); supernatants were collected after 72 hours, and the cytokines IL-5, IL-13, IL-10, and IFN-γ were measured by ELISA. Results are expressed for each group of mice as mean ± SEM (n = 5; Fig 1, *A*), as pooled data (Fig 1, *B*), or as a ratio of IL-10 to IL-5 production **(C)**.

of peptide was sufficient to suppress T_H2-mediated airway inflammatory responses to allergen challenge, we assessed the extent of eosinophil infiltration into the BALF. For this purpose, the lungs of Der p 1–challenged mice were lavaged 24 hours after antigen exposure. There was a significant ($P < .05$) reduction of 94% in the extent of eosinophil recruitment into the lungs of mice pretreated intranasally with Der p 1;p111-139 peptide adsorbed to chitosan compared to the control mice treated with saline solution. No reductions in eosinophil recruitment were observed in mice given peptide alone (Fig 3, *A*).

The effect of intranasal peptide treatment on suppression of T_H2 activation and cytokine production in the airways

The T_H2 cytokines IL-4 and IL-5 are associated with eosinophil recruitment and survival in the lungs of allergic asthmatic individuals. We determined whether the loss of eosinophil recruitment was a direct effect of the reduction in levels of these cytokines in the lungs of tolerized mice after allergen challenge. Groups of mice receiving chitosan–adsorbed Der p 1;p111-139 peptide intranasally before systemic immunization showed significant reductions in BALF levels of 73% ($P < .01$) and 65% ($P < .05$) in IL-4 and IL-5, respectively, compared with control mice treated with saline solution. There was no reduction in IL-4 levels and there was a nonsignificant reduction of 48% in IL-5 levels in BALF obtained from mice treated with peptide alone. Intranasal administration of chitosan alone had no effect (Fig 3, *B* and *C*). Eosinophils are also a source of IL-5 in the airways. Reductions in the T_H2 cell cytokine IL-4, which initiates the influx of eosinophils into the airways through upregulation of adhesion molecules on endothelial cells, might more closely reflect changes in eosinophil levels in BALF.

The induction of sustained T-cell tolerance is preceded by transient activation marked by an increase in the levels of IFN-γ production

In several experimental models it has been reported that antigen-specific T-cell activation precedes the induction of peripheral tolerance. We investigated whether the differential effects of intranasally administered soluble peptide and peptide adsorbed to chitosan on the induction of tolerance was due to a failure to prime naive T cells or was instead due to differential effects on T-cell effector function. For this purpose, cervical lymph nodes draining the nasal cavity were removed either 48 or 96 hours after intranasal administration of peptide or peptide-chitosan and cells stimulated with increasing doses of Der p 1 p111-139 peptide. Peptide antigen–reactive T cells isolated from mice treated with peptide or peptide-chitosan were highly activated at 48 hours, showing dose-dependent increases in proliferation on in vitro stimulation with peptide. However, in mice given peptide-chitosan, reactive T cells exhibited 2- to 3-fold increases in IFN-γ production on stimulation with equimolar concentrations of peptide compared to peptide-treated mice. This was consistent over a dose range of peptide, indicating that it was not due to differences in TCR activation thresholds. Furthermore, at lower concentrations of peptide, IL-5 production was suppressed in T cells isolated from peptide-chitosan–treated mice (Fig 4, *A*). By 96 hours, T cells obtained from all groups of mice were nonresponsive to in vitro restimulation with peptide; there was a loss in both proliferative and cytokine responses (Fig 4, *B*). Chitosan itself failed to activate T cells.

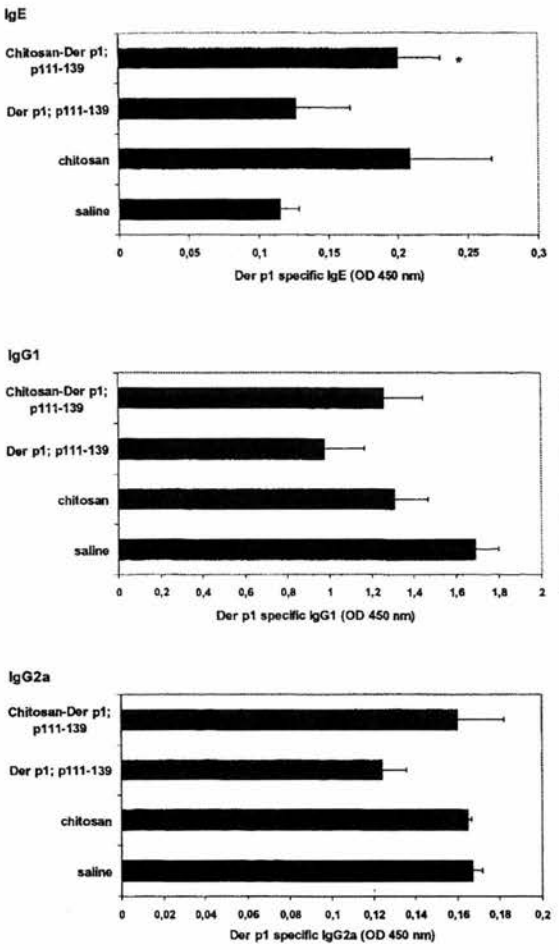

FIG 2. The induction of peripheral T-cell tolerance by mucosal peptide delivery has little effect on Der p 1 specific antibody levels in sera. Levels of Der p 1–specific IgE, IgG1, and IgG2a in sera were determined through use of ELISA. Serial dilutions of sera were set up in duplicate, and the results are presented as mean ± SEM (n = 5) for each group of mice; significant differences between treatment and control groups are indicated (*$P < .05$).

DISCUSSION

Here we have investigated, using a murine model of pulmonary inflammation, whether the intranasal administration of Der p 1;p111-139, a peptide covering an immunodominant T-cell epitope of Der p 1, could protect against the induction of T_H2-mediated airway inflammatory responses to the Der p 1 allergen. A previous study demonstrated that intranasal administration of Der p 1 p111-139 peptide protected against T_H1-mediated delayed-type hypersensitivity responses.[17] We demonstrate that peptide alone was insufficient to prevent induction of T_H2-mediated airway inflammatory responses unless it was adsorbed to chitosan, a compound that enhances uptake of antigen across the airway mucosa. The induction of tolerance in the antigen-specific T_H2

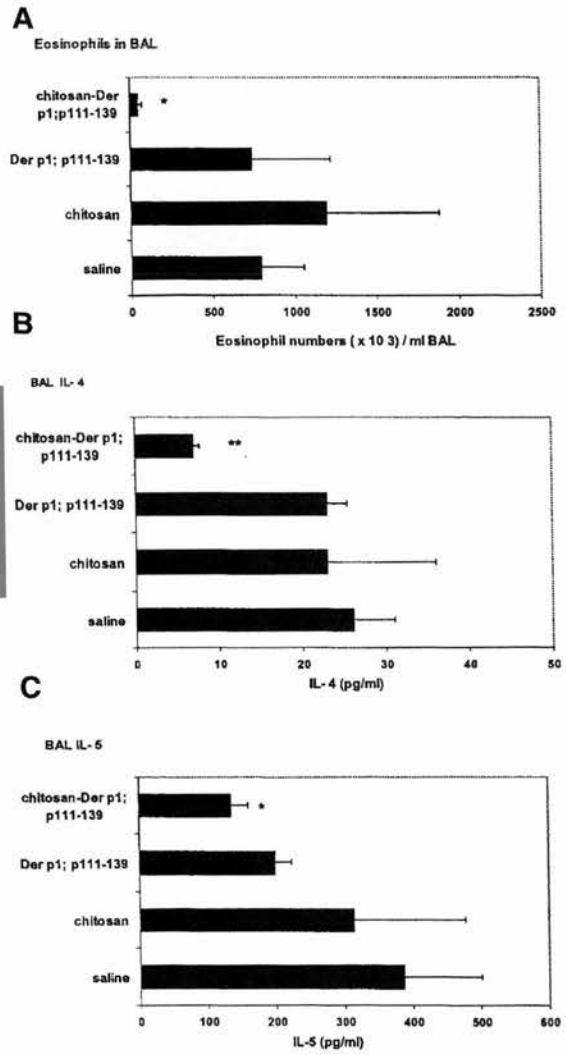

FIG 3. Effect of mucosal delivery of peptide on eosinophil recruitment and cytokine levels in BALF 24 hours after final challenge of the airways with Der p 1. Eosinophil levels in BALF were determined by Diff-Quik staining, and each number was calculated as a percentage of the total cell population per milliliter of BALF **(A)**. Levels of the T_H2 cytokines IL-4 **(B)** and IL-5 **(C)** in BALF were measured through use of quantitative ELISA. These data are presented as means ± SEMs (n = 5) and significant differences between the treatment and control groups are indicated (*$P < .05$; **$P < .01$).

population was preceded by transient increases in proliferation and IFN-γ production with suppression of IL-5.

Changes in the rate and dose of antigen delivery might affect the type of effector T-cell responses generated. The use of peptide adsorbed to chitosan, a compound widely used for controlled mucosal drug delivery,[18] might influence the kinetics of APC activation in the mucosa.[5,19] Changes in the density and affinity of MHC peptide complexes, the expression of costimulatory molecules and cytokines produced by the APCs during T-cell priming, differentially regulate the induction of T_H1 and T_H2 responses.[20,21]

In vitro studies have demonstrated that qualitative and quantitative differences in antigen recognition might affect the affinity of interactions between the TCRs and their ligands and, consequently, the effector response generated. High-affinity TCR–MHC class II peptide interactions prime T_H1 responses, whereas low-affinity interactions prime T_H2 responses.[22] The selective priming of T_H2 responses to inhalant allergens by pulmonary DCs[23] (pDCs) might therefore reflect not only differences in the cytokine milieu in the airways but also the low levels of antigen exposure and consequentially lower-affinity TCR–MHC class II–peptide interactions. Desensitization therapy, which involves the administration of increasing doses of allergen to allergic patients, is associated with both a shift in cytokine profile of allergen-reactive T cells from a T_H2-like to a T_H1-like response and a shift in the induction of T-cell tolerance.[24-26] The transient increase in IFN-γ levels produced by hyperactive T cells isolated from peptide-chitosan–treated mice might be influential in the initial phase of tolerance induction. IFN-γ has been shown to play a protective role in the initial phase of tolerance induction in naive animals to inhalant antigen.[27]

Mice that were given (intranasally) peptide adsorbed to chitosan exhibited a reduction in airway eosinophilia, associated with a fall in levels of the T_H2 cytokines IL-4 and IL-5 in BALF, which is suggestive of a loss of $CD4^+$ effector T-cell recruitment and activation in the airways on allergen challenge. This correlated with a loss of IL-5, IL-13, and IFN-γ produced systemically and an increase in the ratio of IL-10 to IL-5 produced locally in the bronchial lymph nodes, on recall with the Der p 1 allergen. This suggests that attenuation of airway eosinophilia was a consequence of tolerance induction in the Der p 1–reactive effector T_H2 population rather than a T_H2/T_H0 to T_H1/T_H0 shift in cytokines.

Eosinophils are principal effector cells in the pathophysiology of allergic asthma.[28] The induction of airway inflammatory responses and recruitment of eosinophils into the airways after allergen exposure is largely initiated by $CD4^+$ T_H2 cells through the production of IL-4 and IL-5. Both mast cells and eosinophils are also respective sources of these cytokines. The localized production of IL-4 in the airways has been shown to enhance expression of adhesion molecules such as VCAM-1 on endothelial cells, leading to the recruitment of eosinophils along a chemotactic gradient. IL-5 is a principal growth factor and chemoattractant for eosinophils, stimulating recruitment of progenitor cells from the bone marrow and stimulating their differentiation, maturation, and survival in the airways.[29,30] A loss of antigen-specific priming of T_H2 effector responses in the bronchial lymph nodes and a reduction in IL-4 and IL-5 levels in the airways might account for attenuation of airway eosinophilia.[31]

In some patients, successful immunotherapy is associated with a shift in effector phenotype from a T_H2/T_H0 to a T_H1/T_H0 cytokine response, which might be achieved either by promoting T_H1 cytokines or by tolerizing T_H2 effector cells, both of which could shift the balance of cytokines. Studies in patients receiving bee venom

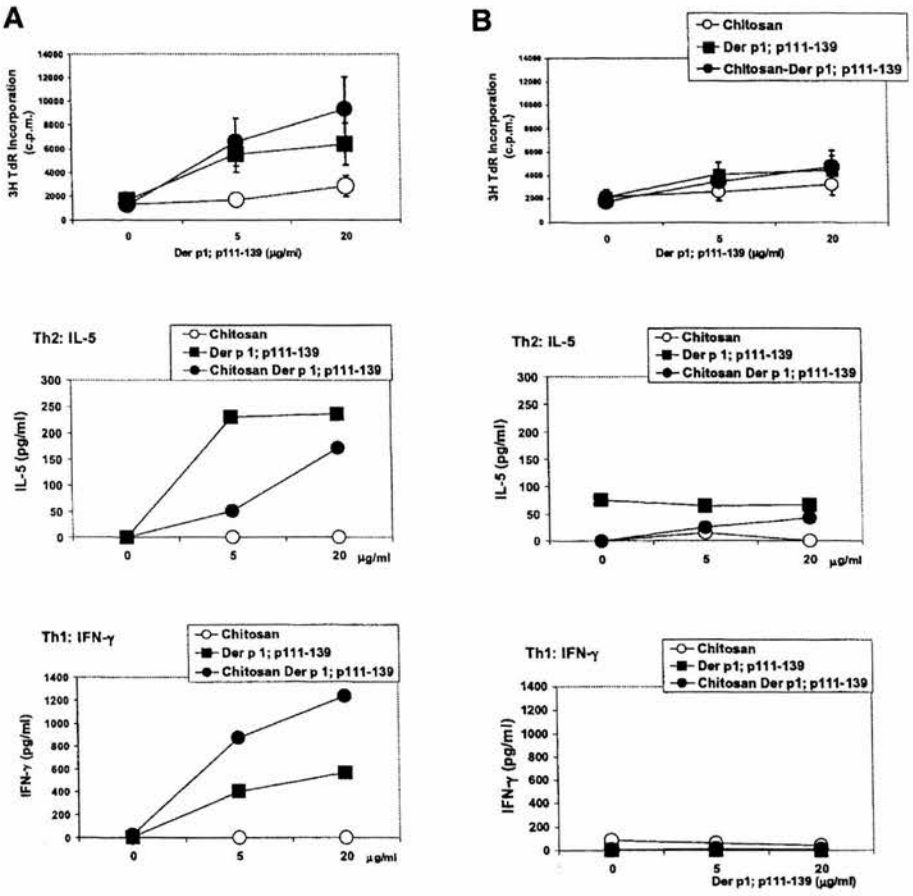

FIG 4. T-cell tolerance induced by mucosal delivery of antigen is preceded by transient activation. Groups of mice that were given (intranasally) peptide, peptide adsorbed to chitosan, or chitosan for 3 consecutive days were killed after 48 **(A)** or 96 **(B)** hours. Cervical lymph nodes were removed and cells were cultured in media or with Der p 1; p111-139 peptide (at 5 or 20 µg/mL); proliferation was determined as 3H TdR incorporation after 72 hours. Culture supernatants were assayed for IL-5 and IFN-γ. These data are expressed as means ± SEMs (n = 5) for each treatment group.

immunotherapy demonstrate that suppression of antigen-specific T-cell proliferation and cytokine production is associated with T-cell anergy induction. Antigen-specific proliferation and T_H1 (IFN-γ) cytokine synthesis, but not T_H2 (IL-4, IL-5, IL-13) cytokine synthesis, could be restored by exogenous IL-2 and IL-15.[26] T-cell anergy was associated with increased levels of IL-10 production, primarily by antigen-specific $CD4^+CD25^+$ T cells in the periphery and subsequently by B cells and monocytes.[32] IL-10 is an immunoregulatory cytokine that has been implicated in the reversal of T_H2-mediated pathology, inhibiting both expansion and cytokine production by effector T_H2 cells and the recruitment of eosinophils into the airways. A potential role for IL-10 in mucosal tolerance induction was first suggested by studies that demonstrated an increase in IL-10 produced by antigen-specific T cells after the intranasal administration of an immunodominant epitope of myelin basic protein.[33] However, until now a role for IL-10–producing T regulatory cells in the induction and maintenance of peripheral tolerance to inhalant aeroallergen had not been shown. The immunoregulatory cytokine IL-10, produced by pDCs after exposure of the airways to soluble antigen, might play a role in downregulating airway inflammatory responses. Production of IL-10, in conjunction with the expression of a mature phenotype by pDCs on migration to the bronchial lymph nodes where antigen is presented to naive T cells, might be instrumental in the induction and maintenance of tolerance to environmental antigens encountered in the respiratory mucosa. pDCs obtained after mucosal exposure to soluble antigen from wild-type mice, but not from IL-10 knockout mice, were shown to adoptively transfer tolerance to naive mice and, in vitro, to prime IL-10–producing $CD4^+$ Tr1 cells.[34] IL-10 is now recognized as a growth factor for a population of $CD4^+CD25^+$ Tr1 cells,[35] originally identified in the thymus, and is now thought to play a critical role in maintaining peripheral tolerance.[36] There is considerable evidence that pDCs play a critical role in the induction of tolerance to inhalant antigen and in the generation of Tr1

cells. Our data suggest that these antigen-specific Tr1 cells might play an important role in maintaining tolerance through production of IL-10 in lymph nodes draining the airways after antigen challenge. IL-10 induces a tolerogenic phenotype in DCs, which correlated with a failure to undergo maturation and upregulation of the costimulatory molecules CD80, CD86, and CD40.[37] The production of IL-10 might therefore account for the observed bystander suppression with a loss of responses not only to the Der p 1;p111-139 epitope but also to minor T-cell epitopes within Der p 1.

In conclusion, we demonstrate that the mode of peptide delivery to the airway mucosa might be fundamental to the induction of sustained T_H2 tolerance to inhalant allergen and reductions in airway eosinophilia. The increase in IFN-γ production and suppression of IL-5 during the initial activation phase and the production of IL-10 in the bronchial lymph nodes following antigen exposure might be instrumental in maintaining a tolerogenic state. Within the limitations of the model system used, these studies give some indication of parameters that might be of importance in designing immunotherapeutic approaches for allergy.

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Suppression of allergen reactive Th2 mediated responses and pulmonary eosinophilia by intranasal administration of an immunodominant peptide is linked to IL-10 production

Gillian Hall [a], Christian G. Houghton [b], Janne Uldal Rahbek [b], Jonathan R. Lamb [a], Elizabeth R. Jarman [a,*]

[a] *Respiratory Medicine Unit, Immunobiology Group, Rayne Laboratory, MRC Centre for Inflammation Research, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, UK*
[b] *ALK-Abello A/S, Hoersholm, Denmark*

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Abstract

The potential to induce systemic tolerance following exposure of the airway mucosa to soluble antigen, may be applied therapeutically for the treatment of allergic disease. Since the use of allergen can trigger IgE mediated inflammation, we investigated whether mucosal delivery of a peptide, containing an immunodominant epitope of the Der p1 allergen of house dust mite, can lead to $CD4^+$ Th2 cell tolerance and thus protect against airway inflammatory responses to inhalant allergen. The administration of microencapsulated peptide to the nasal mucosa of mice, protected against airway inflammation, with significant reductions in eosinophil infiltration into the airways following allergen challenge. Der p1 specific antibody levels in sera were not modulated. Allergen reactive $CD4^+$ T cells expressed a tolerized phenotype, with reduction in levels of the cytokines, IL-5, IL-13 and IFN-γ although IL-10 levels were increased. The mucosal administration of a peptide containing an immunodominant region of an allergen can protect against the induction of systemic and local inflammatory responses to allergen challenge.

Keywords: Allergy; In vivo animal models; Microencapsulated antigen; Immunoregulation

1. Introduction

Allergic asthma is a disease characterized by elevated serum IgE levels and the development of chronic bronchial inflammation, airway hyper-responsiveness and mucus hypersecretion. $CD4^+$ T cells expressing the Th2 cytokines IL-4, IL-5, IL-9 and IL-13 have been identified in the airways of asthmatics [1]. These cytokines are primarily responsible for the disease pathology, which is caused by the influx of mast cells and eosinophils into the airways following exposure to environmental allergens [2].

Specific immunotherapy (SIT), which involves the injection of increasing doses of allergen, can lead to a reduction in clinical symptoms and prevent disease progression. However, the inherent risk of inducing potentially lethal anaphylactic reactions has limited the use of SIT in patients with allergic inhalant asthma. Cross-linking of FcεR on effector cells by IgE-allergen complexes causes the release of preformed mediators responsible for acute immediate inflammatory responses. This has lead to an interest in identifying alternative approaches towards immunotherapy. Successful therapy is associated with increasing levels of IgG4 blocking antibodies and reductions in numbers of mast cells and eosinophils at sites of inflammation [3,4]. The underlying mechanism leading to desensitization is less clearly understood. Recent studies demonstrating augmented levels in IL-10 production, suggest a role for immune regulation and tolerance, rather than the induction of Th1 cytokines.

Abbreviations: AHR: airway hyper-responsiveness; BAL (F): bronchoalveolar lavage (fluids); LN: lymph node; Der p1: *Dermatophagoides pteronyssius* spp.; EPO: eosinophil peroxidase; i.n.: intranasal (ly); PAMP: pathogen associated molecular patterns; SIT: specific immunotherapy

* Corresponding author. Present address: Biological Allergy Research, ALK-Abello A/S, Boege Aue 6-8, DK-2970 Horsholm, Denmark. Tel.: +45-4574-7793; fax: +45-4574-8642.
E-mail addresses: eja@dk.alk-abello.com, eja@dk.alk.abello.com (E.R. Jarman).

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Peripheral tolerance would account for reductions in antigen specific lympho-proliferative responses and IL-4 production. This may result in a subsequent shift in the cytokine milieu with a relative increase in levels of the Th1 cytokines, leading to further reductions in Th2 mediated responses [5–7].

In an effort to enhance the efficacy of therapy, studies have attempted to address the use of different routes of allergen administration, which influence the immune response generated. Studies on autoimmune disease have demonstrated that the mucosal administration of antigen via either the oral or intranasal route, in the absence of inflammatory signals, can protect against disease induction or exacerbation following parenteral challenge with antigen [8,9]. The mechanisms underlying mucosal tolerance in Th1 disease models differ depending on the route of administration. Whereas, oral delivery leads to the induction of a regulatory population of Th3 cells secreting TGF-γ and IL-4, administration of antigen via the intranasal mucosa is thought to lead to the induction of a regulatory Tr1 population, which produce IL-10. Clinical studies are currently addressing the efficacy of sublingual (oral) immunotherapy in the treatment of allergies [10]. However, little is understood about the effect of localized intranasal administration of allergen on targeting airway inflammatory responses. In most individuals, soluble proteins delivered to the nasal mucosa do not invoke an immune response, but induce a state of tolerance. This phenomena has been demonstrated by earlier studies which demonstrated that exposure of naive mice to aerosolized allergen protects against induction of asthma [11]. Studies using murine models of asthma have shown that the prophylactic intranasal administration of a recombinant allergen of birch pollen or long peptides covering the major allergen of bee venom, prior to parenteral immunization with allergen, suppressed systemic Th2 responses and IgE production [12,13]. These studies did not investigate airway inflammatory responses, which may be exacerbated following cross-linking of effector cell bound IgE by allergens, and release of inflammatory mediators. To reduce the risk of eliciting IgE mediated inflammatory responses, clinical studies have addressed the use of peptides in therapy. While clinical studies have demonstrated success in the use of this approach, the induction of late phase asthmatic responses in a proportion of patients following intradermal injection of peptides is a concern [14–16].

To understand the mechanism underlying mucosal tolerance, we have investigated whether Th2 mediated airway inflammation can be modulated by the intranasal administration of peptides. We demonstrate, using a murine model of pulmonary allergic inflammation, that the mucosal delivery of an immunodominant peptide of Der p1, can protect against the induction of Th2 mediated airway inflammation and eosinophilia, in mice challenged with the whole Der p1 allergen. Furthermore, we show that IL-10 plays a critical role in suppressing Th2 responses, including production of the cytokines IL-4, IL-5 and IL-13, which are associated with the pathology of asthma.

2. Materials and methods

2.1. Animals

C57BL/6 mice, 6–8 weeks of age, were used. Animals were purchased from Harlan, UK. Animal care and experimental procedures were conduced in accordance with the animal ethics regulations of the Home Office, UK.

2.2. Antigens

The house dust mite allergen Der p1 was affinity purified from spent mite medium using the monoclonal antibody 4C1 (INDOOR biotechnologies Ltd., UK) [17]. The synthetic peptide Der p1; p111–139 (FGISNYCQIYPPNANKIRELAQPQRYCR) were synthesized with a free carboxyl terminus and HPLC purified (Advanced Biotechnology Centre, Imperial College School of Medicine at Charing Cross Hospital, London). MEA-p111–139 particles were kindly provided by ALK-Abello (Horsholm, Denmark).

2.3. Immunization and tolerance induction

The following treatment regimes were used. Mice were lightly anaesthetized under ether and treated by the intranasal (i.n.) administration, on five consecutive days, of a 20 μl volume of endotoxin free saline containing 100 μg of either peptide Der p1; p111–139, or MEA-Der p1; p111–139 particles (Fig. 1). The control groups received either saline alone or empty MEA particles. In the prophylactic regime, mice were immunized 14 and 16 days after the last i.n. treatment by the intraperitoneal injection of Der p1 (10 μg) in saline absorbed to an equal volume of aluminum hydroxide adjuvant (Imject Alum; Pierce, Rockford, IL). On days 41 and 43, mice were challenged by intratracheal instillation of Der p1 (20 μg) into the airways in 50 μl saline. For the therapeutic regimen, mice were primed by a subcutaneous injection, followed by seven intraperitoneal injections, at 2 day intervals of Der p1 (10 μg in saline). Eight days after the last injection, groups of mice were treated by the intranasal administration of a 20 μl volume of endotoxin free saline containing 100 μg of either peptide Der p1; p111–139, MEA-Der p1; p111–139 particles, Der p1 allergen, or MEA-Der p1 (Fig. 1). The control groups received either saline alone or empty MEA particles. After 2 weeks, the airways were challenged three times, by instillation of Der p1 (20 μg in 50 μl saline) at 2 day intervals. Mice were sacrificed after 24 h, using a lethal dose of avertin. To determine antibody levels in sera, mice were bleed from the tail vein prior to sacrifice. Blood samples were centrifuged at 2000 × *g*, sera collected and stored at −70 °C.

2.4. Cytokine production

Single-cell suspensions were prepared from spleen or lymph nodes by teasing the tissue apart and passing it

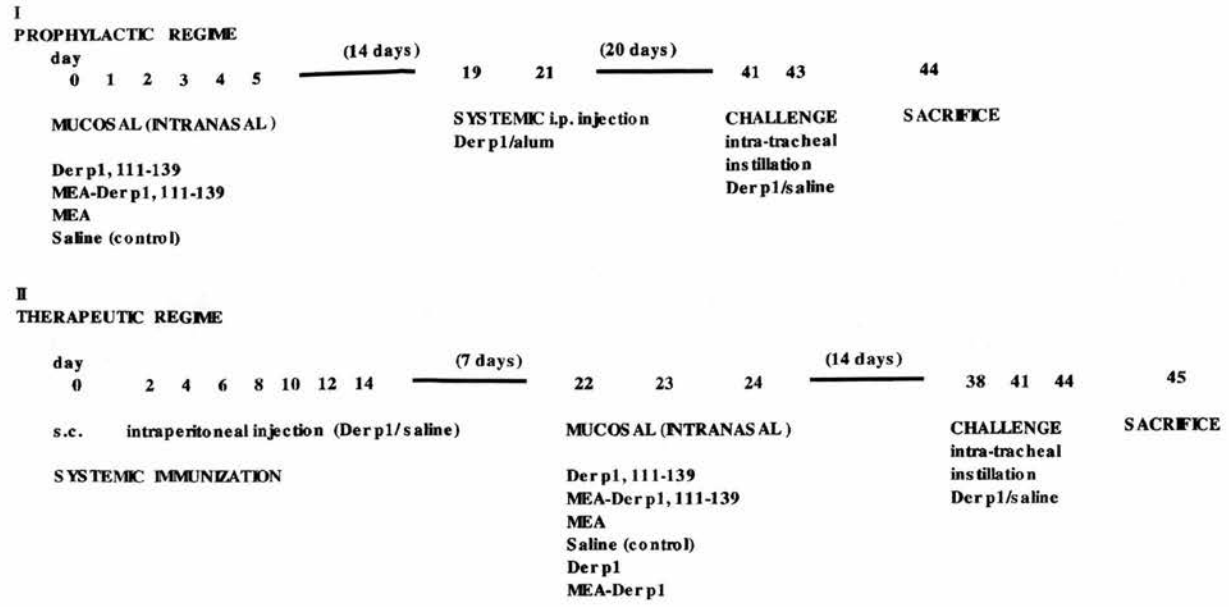

Fig. 1. (I) Prophylactic immunization regimen; groups of naive mice were treated intranasally on five consecutive days with 20 μl saline containing 100 μg Der p1; p111–139 peptide either alone, or encapsulated in MEA particles. Control mice received saline or MEA particles alone. On days 19 and 21, mice were immunized with Der p1/alum and after 20 days, challenged twice by instillation of 20 μg Der p1 into the airways. After 24 h mice were sacrificed. (II) Therapeutic regimen; mice were sensitised as shown by multiple intraperitoneal injections with Der p1 in saline. After 7 days, mice were treated intranasally with either Der p1 or the Der p1; p111–139 peptide either alone or encapsulated in MEA particles. Controls received MEA particles or saline. Mice were then challenged by instillation of Der p1 directly into the airways and sacrificed 24 h later.

through a fine wire mesh to remove cell debris. Cells were cultured in RPMI 1640 medium supplemented with 5% foetal calf serum (FCS; GIBCO), 50 μM 2-mercaptoethanol, 2 mM L-glutamine and 10 U penicillin/streptomycin (GIBCO, Paisley, UK). Cells (5×10^5 per well) were cultured in 96-well flat-bottomed plates (Costar) with either Der p1 or peptide Der p1; p111–139 (10 μg/ml). Control wells contained medium alone, OVA (10 μg/ml) or were coated with anti-CD3 and anti-CD28 (5 μg/ml). After 72 h supernatants were collected, stored at −70 °C and assayed in duplicate for the presence of the cytokines; IL-4, IL-5, IL-10, IL-13 and IFN-γ using ELISA. Antibodies were purchased and used according to the instructions of the manufacturer (IL-4, IL-10, IL-13; R& D Systems, UK, or IL-5, IFN-γ Pharmingen, San Diego, CA).

2.5. Measurement of eosinophil levels in bronchoalveolar lavage

Immediately after sacrifice, the trachea were exposed and cannulated with 27-gauge silicon tubing attached to a needle and syringe. The lungs were instilled through the trachea with 500 μl of cold PBS and the BAL fluid withdrawn. The volume of BAL fluid collected was measured for each sample. To determine BALF cell numbers and composition, samples were centrifuged (700 × *g*, 5 min, 4 °C), cells were washed (PBS/10% FCS), counted and re-suspended at 1×10^6 ml^{-1}. BALF (1×10^5) cells in 100 μl were cytospun onto poly(L-lysine)-coated slides (3 min at 300 rpm). The slides were air-dried, fixed in methanol and stained using Diff/Quick. To determine cytokine levels in BALF, samples were stored at −70 °C. All cytokines were analyzed by ELISA.

2.6. Extraction and measurement of eosinophils numbers in lung tissue

Lungs were weighted and homogenized in Hanks balanced salt solution (HBSS; Gibco) containing 10 mM HEPES (pH 7.4). Following red blood cell lysis, cells were re-homogenized in HBSS containing 0.5% hexadeclytrimethylammonium bromide (HTAB; pH 7.4) and freeze thawed in liquid nitrogen. Levels of eosinophil peroxidase in lung homogenates were determined using the *O*-phenylenediamine substrate (Sigma; OPD 1.5 mM, H_2O_2 6.6 mM; pH 8). The reaction was stopped using H_2SO_4 (4 M) and plates read in a MRX microplate reader at 490. Data was analyzed using Revelation software.

2.7. Determination of Der p1 specific antibody levels in sera

Antibody levels in sera, were determined by ELISA. Unless otherwise indicated serum samples and antibodies were diluted in PBS/1% BSA and wells washed between each incubation step with PBS/0.05% Tween-20. Round

bottomed 96-well microtitre plates (Costar) were coated overnight at 4 °C with either rat anti-mouse IgE (R35-72; Pharmingen), or with Der p1 (5 μg/ml), diluted in carbonate buffer (0.1 mM $NaHCO_3$, pH 8.2). After washing, non-specific binding was blocked by incubating wells PBS/3% BSA for 2 h at 37 °C. Plates were washed and serial dilutions of serum samples added in duplicates (IgE 1:5, 1:10, 1:20; IgG1 1:50, 1:100, 1:500; IgG2a 1:10, 1:20, 1:50). The plates were incubated overnight at 4 °C. After washing, biotinylated rat anti-mouse IgG1 (LO-MG1-2; Serotec) or rat anti-mouse IgG2a (Lo-MG2a-7; Serotec) was added at 1000 ng/ml and plates incubated for 2 h at room temperature. To determine Der p1 specific IgE levels in sera, Der p1 (10 μg/ml) was added and plates incubated at 37 °C for 60 min. After washing, plates were incubated for a further 45 min with biotinylated 4C1 (1:1000 dilution). Bound biotinylated antibodies were detected by incubating with streptavidin-peroxidase for 45 min at room temperature and visualized using the TMB substrate. Plates were read in a microtitre reader at 450 nm using the MRX microplate reader and Revelation software.

2.8. Statistics

Data represents mean values ± S.E.M., with five mice per group. Differences between mean values were analyzed by one way analysis of variance (ANOVA) using the Dunnett Multiple comparison parametric test and Instat software. $P < 0.05$ were considered statistically significant.

3. Results

3.1. Prophylactic effects of intranasal administration of an immunodominant peptide on Der p1 allergen induced airway eosinophilia

Eosinophil infiltration of the airways is a predominant feature of allergic asthma. To investigate whether the intranasal administration of a peptide covering an immunodominant T cell epitope of Der p1; p111–139, could protect against the induction of Th2 mediated airway inflammatory responses to Der p1, eosinophil numbers in the BAL and lung tissue were determined. Groups of mice were administered intranasally either Der p1; p111–139 peptide (5 × 100 μg) alone or encapsulated in MEA particles on five consecutive days. Mice were then immunized systemically and challenged by instillation of Der p1 into the airways (Fig. 1). Eosinophil numbers in the lung tissue and BAL were determined 24 h after allergen challenge. A significant reduction ($P < 0.05$) of 66.3% in eosinophil numbers in the BALF was observed in mice treated intranasally with MEA encapsulated Der p1; p111–139 peptide, as compared with the control group receiving PBS (Fig. 2A). This correlated with a significant ($P < 0.01$) reduction of 54.4% in eosinophil numbers in the lung tissue (Fig. 2B). No reduction in eosinophil levels in the BAL or lung tissue were observed in mice administered Der p1; p111–139 peptide alone (Fig. 2). The suppression of eosinophilia was antigen dependent since empty MEA particles had no effect. Preferential uptake of MEA-peptide by immunosuppressive APC such as pulmonary dendritic cells (DC) or alveolar macrophages may account for reductions in Th2 mediated inflammation. Alternatively, the kinetics of peptide released systemically from MEA particles may, by altering the frequency and duration of TCR ligation, reduce the activation threshold required for T cell tolerance induction.

3.2. Cytokine levels in the BALF

Allergic asthmatics have elevated levels of the cytokines IL-4 and IL-5 present in the BALF [1]. These cytokines play a critical role in initiating and sustaining airway inflammation and eosinophilia. To determine whether reductions in eosinophil numbers in the lung and BALF of MEA-peptide intranasally treated mice, were due to a loss of allergen reactive $CD4^+$ T helper activity, levels of these cytokines were measured in BALF of mice 24 h after challenge with Der p1. Marked reductions in levels of IL-4 (35%) and IL-5 (62%) were observed in the BALF of mice treated i.n. with MEA encapsulated Der p1; p111–139, as compared with control PBS treated mice. Reductions in IL-5 levels (48%), were also observed in mice treated intranasally with Der p1; p111–139 peptide alone. No parallel reduction was observed in IL-4 levels, suggesting that other factors in addition to IL-5 influenced eosinophil levels (Fig. 3).

3.3. Mucosal tolerance leads to reductions in antigen specific production of both Th1 and Th2 cytokines, produced systemically

To investigate whether reductions in eosinophilia was due to a Th2 to Th1 cytokine shift, splenocytes obtained from individual mice were cultured in the presence of Der p1 or the immunodominant peptide Der p1; p111–139. Cytokine levels in the supernatants were assayed after 72 h (Fig. 4). In comparison with the control saline treated group, Der p1 reactive T cells obtained form mice administered MEA-Der p1; p111–139 had reduced levels of both the Th2 (IL-5 (69%), IL-13 (67%)) and Th1 (IFN-γ (82%)) cytokines. Reductions in airway eosinophila were, therefore, due to a loss of antigen specific T cell responsiveness and eosinophil recruitment, rather than a Th1 shift in cytokine production. The loss of T cell responsiveness to intact Der p1, following mucosal tolerance induction with an immunodominant T cell epitope (Der p1; p111–139), may be due to linked suppression resulting in a loss of responsiveness to minor T cell epitopes within Der p1. Peptide alone, failed to tolerize Der p1 reactive T cells, suggesting that MEA particles enhanced the tolerogenicity of the peptide. Whereas, the inhibitory effect of MEA-peptide on Der p1 specific responses is antigen dependent, the significant reduction in IFN-γ levels

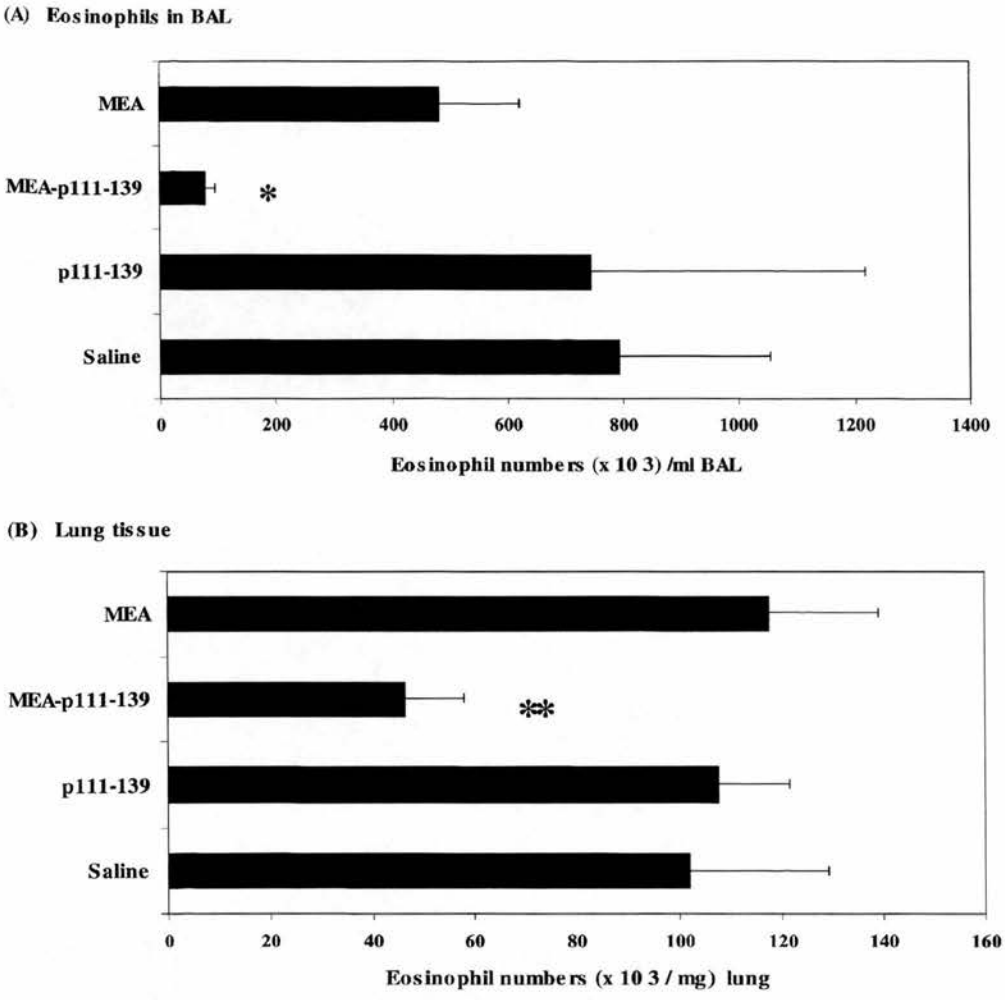

Fig. 2. The effect of i.n. treatment with Der p1; p111–139 on levels of eosinophils in BALF and lung tissue of mice sensitised and challenged with Der p1. (A) Eosinophil numbers in BALF were determined by Diff/Quick staining of cytospins. For each treatment group, cell number were determined as a percentage of total cell numbers per ml BALF and expressed as mean ± S.E.M. Differences in means were significant ($^{*}P < 0.05$). (B) Eosinophil numbers in lung tissue were determined by measuring eosinophil peroxidase levels in lung homogenates. The data is presented as mean eosinophil numbers ± S.E.M. for each group of mice. Differences in means were significant ($^{**}P < 0.01$).

($P < 0.001$) may be largely due to a non-specific effect of MEA particles. Background cytokine levels were negligible (Fig. 4).

3.4. Antibody levels and isotype are not significantly suppressed by intranasal peptide treatment

To determine whether the i.n. administration of a single T cell epitope peptide could influence B cell responses, Der p1 specific antibody levels in sera were measured by ELISA. Der p1 specific IgE, IgG1 and IgG2a levels were not reduced in mice receiving MEA-peptide as compared with control mice, although there was a 42% reduction in Der p1 specific IgG1 levels, in sera obtained from Der p1; p111–139 treated mice. Mice treated with MEA particles alone, had increased levels of specific IgE in sera, which may be linked to the non-specific reduction in IFN-γ production (Fig. 5).

3.5. Pulmonary eosinophilia is reduced following local allergen challenge and correlates with an increase in the ratio of antigen induced IL-10:IL-5

We investigated whether or not IL-10 plays a role in the intranasal peptide induced Th2 tolerance to Der p1 allergen. Supernatants obtained form spleen or pooled bronchial lymph node cells, were cultured with Der p1 or Der p1; p111–139 and assayed for IL-10 production by ELISA. There was an increase in levels of IL-10 production by antigen specific T cells obtained from the draining lymph nodes of mice treated i.n. with MEA-Der p1; p111–139 or peptide Der p1; p111–139 alone, as compared with cells obtained from control mice. IL-10 levels produced by splenocytes obtained from intranasally tolerized mice were not significantly greater than in the control group. Comparisons of the ratio of IL-10 to IL-5, revealed a significant increase in the

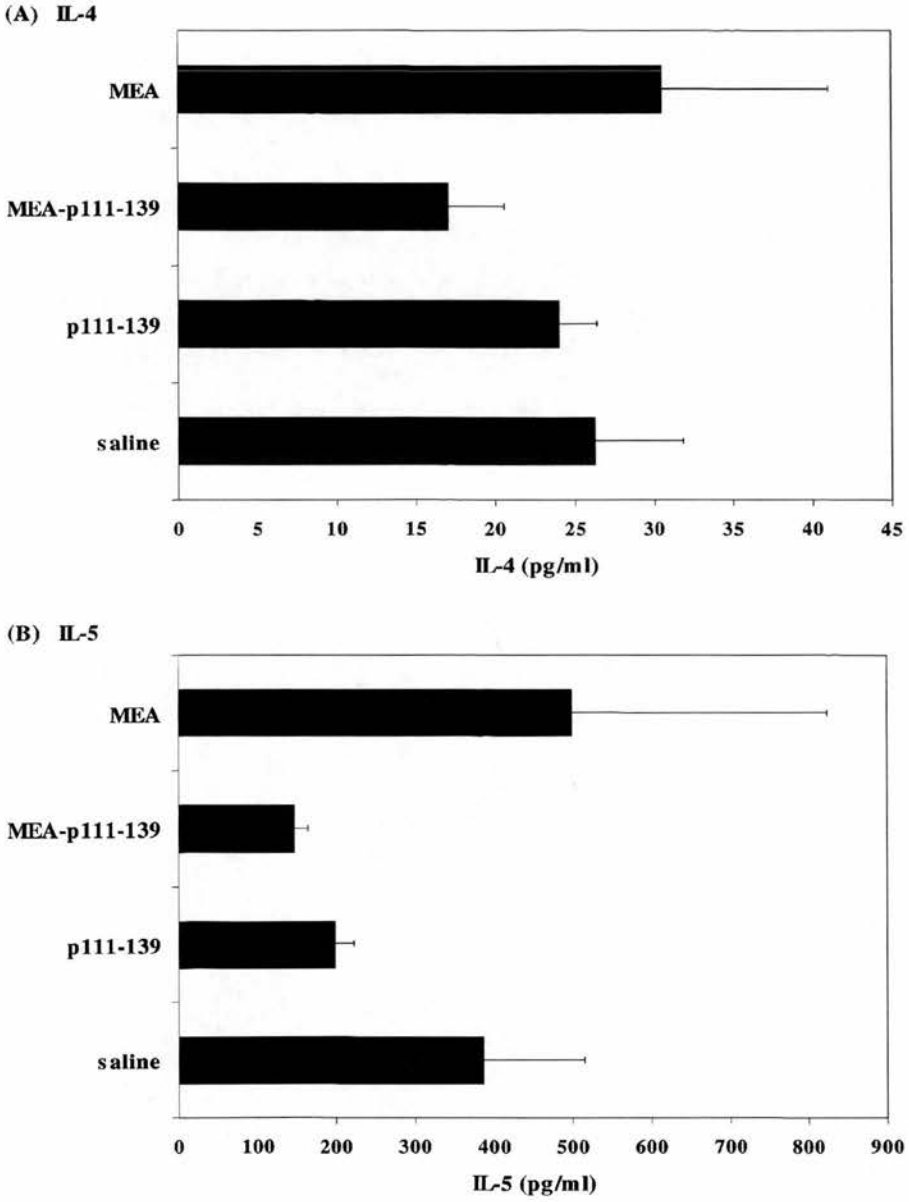

Fig. 3. Reduction in levels of the Th2 cytokines IL-4 and IL-5 in BALF of mice treated with MEA-peptide (Der p1; p111–139), correlated with reduced numbers of eosinophils in BALF and lung tissue. IL-4 (A) and IL-5 (B) in BALF obtained from mice 24 h after challenge of the airways with Der p1 were determined by ELISA. The data is presented as mean ± S.E.M. for each treatment group.

ratio of IL-10, which correlated with a reductions in lung eosinophilia. Increases were observed in cultures of spleen and draining lymph node cells obtained from MEA-peptide treated mice (Fig. 6).

3.6. *Effect of the therapeutic intranasal administration of Der p1; p111–139 peptide to Der p1 sensitized mice*

Studies have demonstrated that the intranasal administration of allergen to mice sensitized systemically, can lead to airway inflammation. We investigated whether the intranasal administration of a Der 1 peptide, using the therapeutic regimen (Fig. 1), leads to reductions or exacerbations in established Th2 mediated response to Der p1. For this purpose, groups of mice primed by systemic immunization with Der p1, were treated intranasaly with peptide (Der p1; p110–131) alone or encapsulated into MEA particles. Therapeutic administration of MEA-peptide or peptide alone did not exacerbate airway eosinophilia in sensitised mice. In contrast, the intranasal administration of Der p1 allergen to sensitised mice, lead to increases in airway eosinophilia of 70% and an increase of 55% in Der p1 specific IgG1 levels in sera ($P < 0.01$). Increases in eosinophilia and specific IgG1 in pre-sensitised mice were not elevated on intranasal administration of Der p1 adsorbed to MEA. Fig. 7A). In contrast, administration of Der p1; p110–131 peptide or MEA-peptide

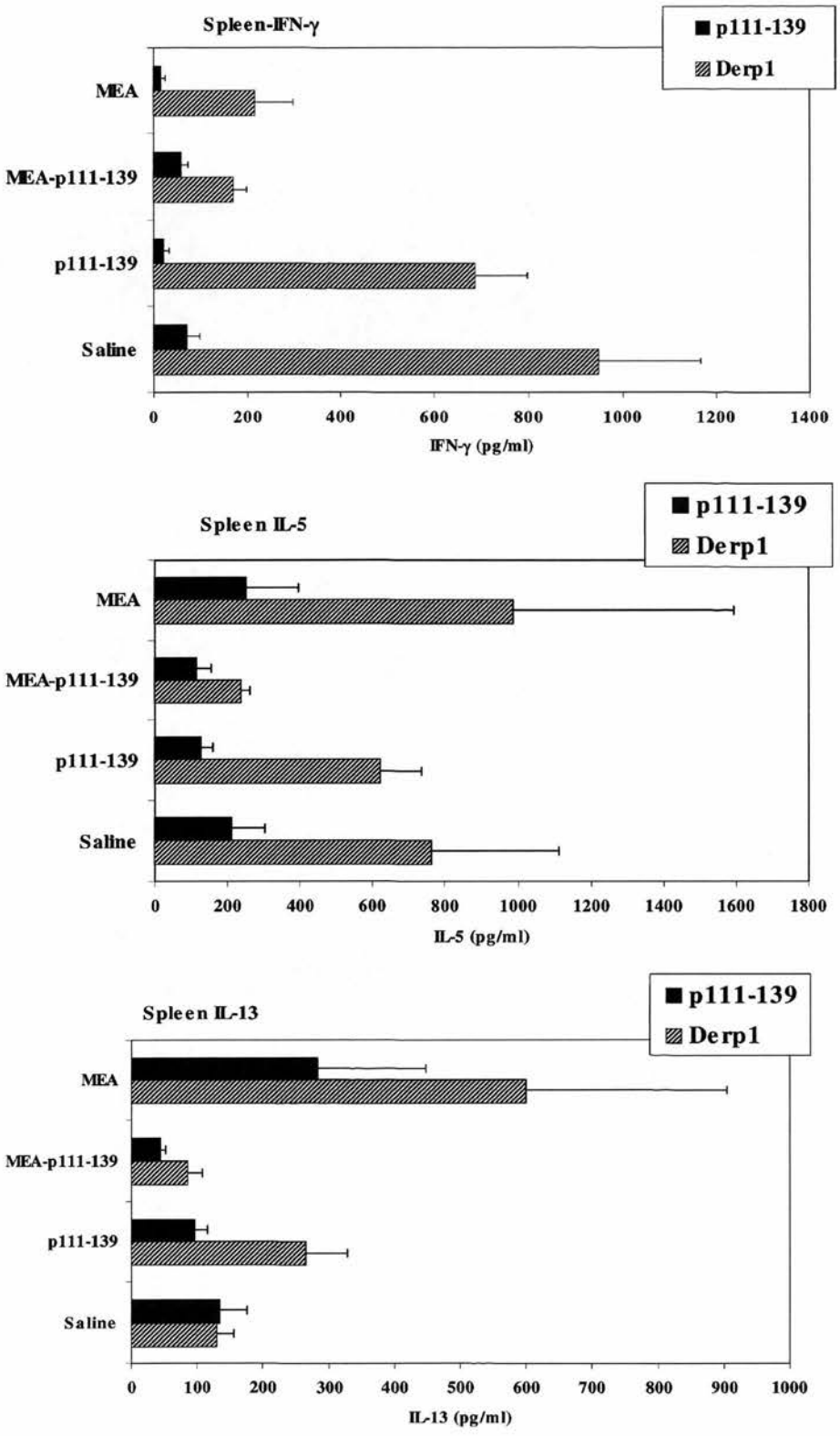

Fig. 4. Reduction in both Th2 (IL-5) and Th1 (IFN-γ) cytokine levels. Splenocytes obtained from individual mice were cultured (5×10^6 per ml) in the presence of either Der p1 or the peptide Der p1; p111–139 (10 μg/ml). Supernatants were collected after 72 h and cytokine levels assayed by ELISA. Cytokines in the supernatants of splenocytes cultured in media alone or an irrelevant antigen were at baseline levels. The data is presented as mean ± S.E.M. for each group of mice. Differences in means were significant ($^*P < 0.001$).

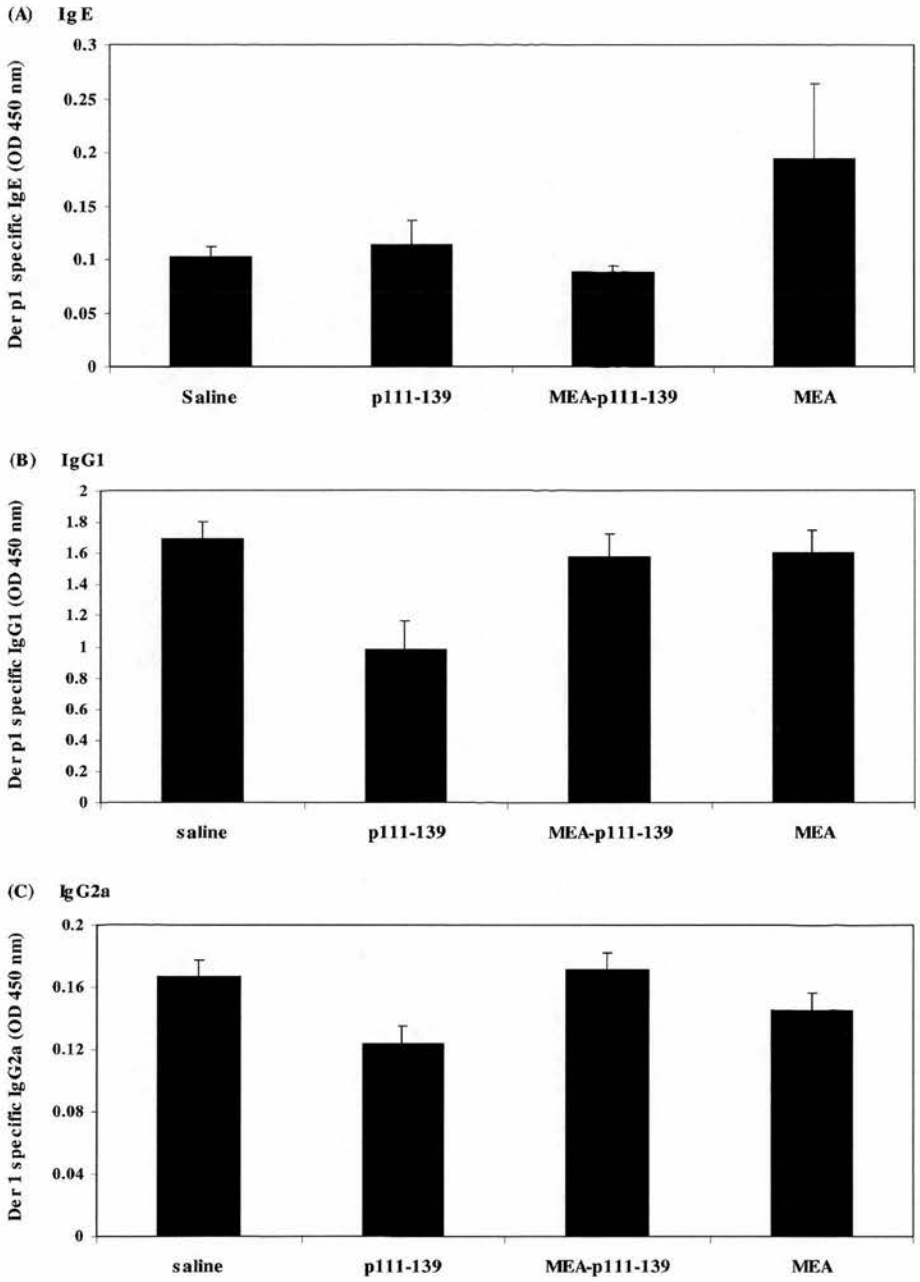

Fig. 5. Der p1 specific IgE and IgG levels in sera. Der p1 specific antibodies in sera were determined on day 45. Antibody levels, measured by ELISA are presented for the following dilutions; specific IgE (1/20 dilution), IgG1 (1/100 dilution) and IgG2a (1/10 dilution). The data is presented as OD 405 nm (mean ± S.E.M.) for each group.

to systemically sensitised mice, lead to non-specific reductions in Der p1 specific IgG1 (60%, 52%) and IgG2 (47%, 59%) levels in sera, as compared to control mice (Fig. 7).

4. Discussion

Allergic asthma is thought to arise following a loss of tolerance to inhaled allergen. The exposure of naive mice to aerosolized allergen has been shown to protect against the induction of allergic responses [11]. A number of groups have demonstrated that exposure of the airway mucosa to soluble allergen by intranasal administration can lead to the induction of tolerance. However, concerns about the potential for triggering IgE mediated inflammatory responses, have prompted investigations on the use of peptides, which contain T cell epitopes in therapy.

Previous studies demonstrated that residues p111–139 of Der p1 contained an immunodominant T cell epitope. Stimulation with a peptide covering this region induced

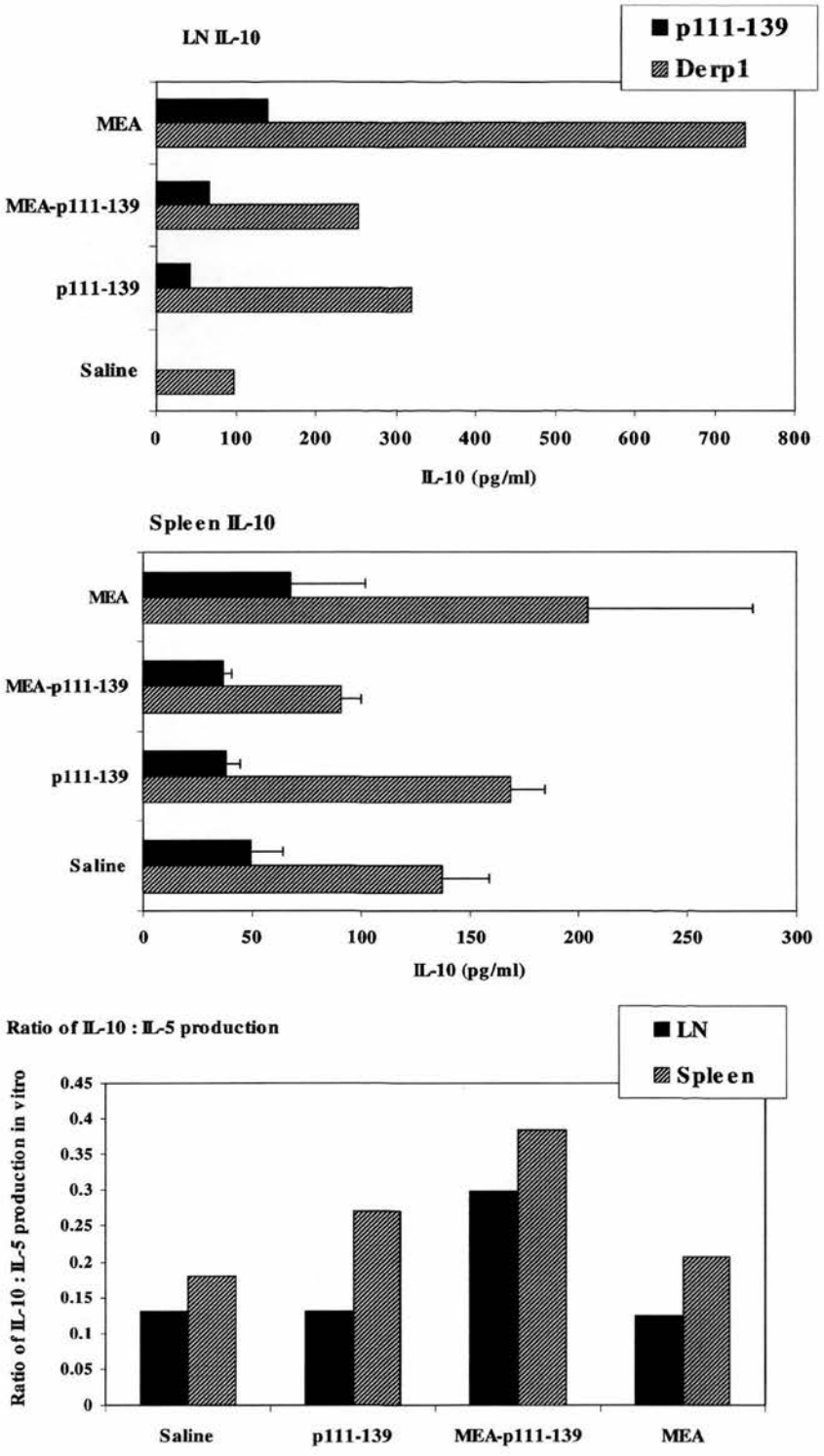

Fig. 6. Intranasal MEA-peptide treatment results in an increase in the ratio of IL-10 to IL-5 produced by Der p1 reactive T cells in vitro. IL-10 and IL-5 levels in supernatants obtained from Der p1 (10 μg/ml) stimulated cells obtained from the spleen or bronchial lymph nodes, were assayed by ELISA. Mean values from individual spleen and pooled lymph node cells were used for each group. The data is presented as the ratio of IL-10 to IL-5 produced by each treatment group.

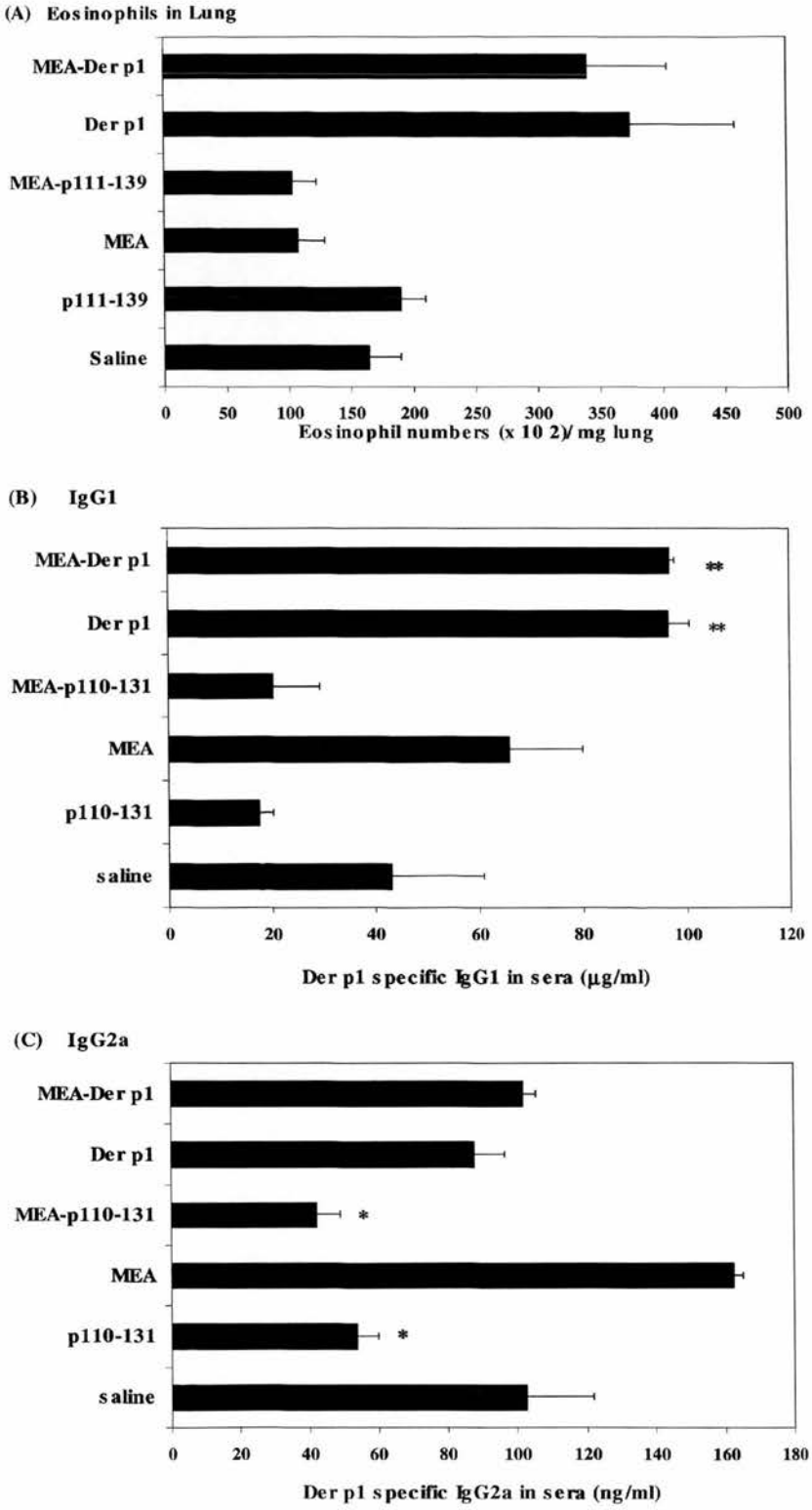

Fig. 7. Therapeutic intranasal administration of Der p1 peptide to sensitised mice causes in reduction in systemic humoral responses but not in airway eosinophilia, following challenge with Der p1. Groups of mice sensitised systemically to Der p1, received three consecutive intranasal administrations of either peptide or Der p1, alone or encapsulated in MEA particles. Control mice received PBS or MEA alone. Eosinophil numbers in the lung were determined using the quantitative eosinophil peroxidase assay (A), Der p1 specific IgG1 (B) and IgG2a (C) levels in sera were determined by ELISA. The data is presented here as mean ± S.E.M. for each treatment group. Differences in mean between the treatment and control saline group are significant ($^{*}P < 0.05$, $^{**}P < 0.01$) using Dunnett multiple comparison test.

lympho-proliferative responses in human T cell clones, and murine T cell lines obtained from Der p1 immunized mice expressing the H-2^b haplotype [17,18]. The intranasal administration of peptide Der p1; p111–139 to naïve mice protected against the induction of Th1 mediated DTH responses, following immunization with Der p1 in CFA [19]. In this study, we demonstrate, using a murine model of pulmonary inflammation that the intranasal delivery of an immunodominant T cell epitope peptide can also protect against the induction of Th2 mediated eosinophilia, which is a principal feature of allergic asthma. Mice administered MEA encapsulated peptide intranasally had significantly reduced numbers of eosinophils in the BAL and lung tissues after inhalation of the Der p1 allergen. Mucosal tolerance is linked to the degree to which the $CD4^+$ T cell population is initially activated, since the intranasal administration of peptide alone, failed to protect against the induction of eosinophilia. Antigen concentration and TCR affinity are critical in determining the strength of initial activation signals that precede T cell tolerance [20,21]. Our data suggest that by using MEA encapsulated peptide, we were able to increase the concentration of peptide released systemically thus strengthening the initial TCR mediated activation signal.

Eosinophil infiltration of the airways is a characteristic feature of allergic asthma. Eosinophils contribute towards airway inflammation not only by the release of inflammatory mediators, but also as antigen presenting cells, capable of amplifying localized Th2 mediated responses [22]. The recruitment and differentiation of eosinophil progenitor cells in the bone marrow as well as the infiltration and survival of eosinophils at sites of inflammation are all regulated by IL-5. Signaling by IL-4 or IL-13 through the IL-4Rα chain is also critical for development of an allergic asthma phenotype [23]. Reductions in levels of the cytokines IL-4 and IL-5 in the BAL fluid of mice challenged with Der p1, may account for reduced eosinophil numbers in the airways of mice administered MEA-peptide intranasally. However, a decrease in levels of IL-5 in the BALF did not result in a reduction in airway eosinophilia in peptide treated mice, suggesting that other factors contributed to the reduction in eosinophil numbers. A separate study, investigating systemic responses generated by the intranasal administration of overlapping peptides from PLA2, demonstrated a shift in cytokine profile, with an increase in the ratio of IFN-γ to IL-4 [13]. Here we fail to demonstrate a Th2 to Th1 shift in cytokine profile by antigen specific cells following successful intranasal therapy. Comparison of levels of cytokines produced by spleen and draining lymph node (data not shown) cells obtained from control or MEA-peptide treated mice, revealed a reduction in levels of the Th2 (IL-5, IL-13) as well as the Th1 (IFN-γ) cytokines. Reductions in allergen specific production of both Th1 and Th2 cytokines were also observed in a separate study using a recombinant allergen of Bet v1 [12]. The general view that Th1 cytokines, by suppressing Th2 responses may protect against progression of allergic disease, is not supported by data, which indicates that elevated IFN-γ levels can exacerbate airway inflammatory responses to allergen [24,25].

Studies involving the intranasal administration of whole allergen, demonstrated that the induction of peripheral T cell tolerance was accompanied by modified B cell responses, with reductions in specific IgE and changes in the ratio of IgG1 to IgG2a levels in sera [12,13]. Data obtained from this study, demonstrate that the prophylactic use of a short linear T cell epitope peptide, while suppressing local Th2 dependent inflammatory responses in the airways, did not greatly influence B cell dependent responses such as antibody levels in sera or isotype. In contrast, the intranasal administration of a T cell epitope peptide p110–131, to mice systemically sensitised with Der p1, lead to a reduction in B cell dependent antibody responses, but failed to suppress airway eosinophilia. There is considerable data from animal models of asthma, which demonstrate that many of the processes of asthma, including eosinophil infiltration of the bronchial mucosa and bronchial hyper-responsiveness are elicited independently of the presence of B cells and IgE, but are dependent on the presence of T cells [26,27]. The induction of tolerance in the allergen specific T cell population by mucosal delivery of peptide containing a T cell epitope is sufficient to cause a reduction in eosinophil levels in the airways, independently of any effect on B cell responses. Failure to significantly downregulate allergen induced eosinophilia following mucosal delivery of peptide to sensitised mice, suggests that it may be more difficult to suppress established Th2 mediated responses, by tolerance induction. This view is supported by a recent publication demonstrating that ongoing Th2 responses in the lungs of mice prevented induction of tolerance to aerosolized OVA [28].

We have attempted to address the mechanism underlying the induction of Th2 tolerance. Linked suppression is thought to account for the loss of responsiveness to all epitopes within a protein, following the induction of T cell tolerance using a single immunodominant epitope. This form of suppression may be mediated via an antigen non-specific cognate interaction with APC [29], possibly involving signaling through Notch ligands and their receptors [30] and/or through the production of immunosuppressive cytokines such as IL-10 and TGF-β. There has been considerable evidence demonstrating that TGF-β plays a role in the loss of responsiveness arising due to the induction of mucosal tolerance [12,31]. Animal studies have indicated that the immunoregulatory cytokine IL-10 is a critical factor in the induction of mucosal tolerance to a Th1 response to autoantigen [32]. Clinical studies have demonstrated an increase in IL-10 production by allergen reactive $CD4^+$ T cells in the periphery following SIT [33]. However, until now, a role for IL-10 in the induction of tolerance to allergen and consequently a reduction in Th2 mediated airway inflammation has not been demonstrated using in vivo models. In this study, we have shown that after prophylactic ther-

apy with peptide alone or encapsulated in MEA particles, there is an increase in levels of IL-10 production following antigen stimulation of cells obtained from lymph nodes draining the airways. Reductions in eosinophil numbers correlated with a parallel reduction in IL-5 production. IL-5 is a critical differentiation and survival factor for eosinophils as well as a major chemoattractant. It is therefore, likely that IL-10 induced tolerance in the allergen reactive $CD4^+$ Th2 population, possibly during antigen presentation in the draining lymph nodes, would result in a loss of localized IL-5 production and consequently in eosinophil levels in the airways. Recent evidence suggests that the production of IL-10 by pulmonary DC may be critical for the induction of mucosal tolerance [34]. IL-10 is a critical growth factor for a population of antigen specific $CD4^+$ CD45RB low $CD25^+$ T regulatory type 1 (Tr1) cells, which are thought to be central to the induction and maintenance of peripheral tolerance [35,36]. The transfer of Tr1 T cell clones, but not Th1 or Th2 clones, at the time of OVA immunization inhibited antigen specific serum IgE responses [37]. Although originally described as a murine Th2 factor, it is now recognized that IL-10 inhibits Th2 mediated responses, including airway eosinophilia [38]. The ability of IL-10 to suppress DC maturation may be central to the induction of T cell tolerance and immunoregulation by Tr1 cells [39,40].

Acknowledgements

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