An analysis of allergen mimotopes.

Stephanie Gunn
B. Sc. (Hons)

This thesis is presented for the degree of Doctor of Philosophy in Microbiology from the University of Western Australia, 2005.
Declaration

The work presented in this thesis was performed solely by myself except where otherwise stated and has not been submitted previously for any other degree.

S. R. Gunn
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The journey has been a hard one, but it has not been a lonely one.
Abstract

The aims of this thesis were to isolate allergen mimotopes from phage display libraries and to examine the potential application of these mimotopes as immunotherapeutic agents. An investigation was also made into the potential blocking activity of monoclonal antibodies. A series of phage display libraries were screened with anti-papain, anti-FLAG and anti-Der p 2 monoclonal antibodies, and potential mimotopes or variants of these proteins isolated. The phage-displayed peptides were analysed both in vitro and in vivo. Immunogenic FLAG variants were isolated, and a putative Der p 2 mouse monoclonal antibody epitope (or part thereof) was identified.

Four anti-papain monoclonal antibodies were produced and assessed for in vivo blocking activity. Three of the antibodies possessed the ability to inhibit lung inflammation when delivered before or after allergic sensitisation; one antibody demonstrated no blocking activity. The antibodies were used to screen phage display libraries for mimotopes. Specific phage clones were isolated only for the antibody that possessed no blocking activity; these clones were antigenic, but not immunogenic, so further experiments could not proceed with these reagents.

Biopanning with the model epitope FLAG was carried out to verify the biopanning procedures used for the anti-papain monoclonal antibodies, and a series of FLAG variants isolated. A strong consensus sequence between the specific clones was observed. This consensus displayed similarity to the canonical FLAG sequence. All of the specific clones were highly antigenic, but only one was demonstrated to be immunogenic. This clone was the most similar to the canonical FLAG sequence.

Potential mimotopes of Der p 2 were selected from two random peptide phage display libraries. These peptides shared a consensus sequence that displayed similarity to residues 69-82 of the Der p 2 primary sequence. All of the specific clones were highly antigenic. Two phage clones demonstrated antigenicity, inducing antibodies cross-reactive to Der p 2. The putative epitope 69-82 of Der p 2 was successfully displayed in monovalent display and used to immunise mice. This fusion was highly immunogenic and produced more consistent results than immunisation with phage-displayed peptides. Further analysis was made by introducing this fusion into a pre-
established murine model of respiratory sensitisation. The fusion protein acted to increase lung inflammation, indicating that the residues 69-82 of Der p 2 constitute a pro-inflammatory sequence.

Taken together, the results of the ability of monoclonal antibodies to inhibit allergic responses and the isolation of mimotopes shows that allergen mimotopes could be used for immunotherapy. The results also show, however, that not all monoclonal antibodies are inhibitory or are readily able to be used to produce immunogenic mimotopes.
Abbreviations

The following abbreviations are used throughout this thesis:

- APC: antigen presenting cell
- BSA: bovine serum albumin
- EDTA: ethylenediaminetetraacetic acid
- cDNA: complementary deoxyribonucleic acid
- CFA: complete Freund’s adjuvant
- Cfu: colony forming units
- DC: dendritic cell
- DELFIA: dissociation enhanced lanthanide fluoroimmunoassay
- ELISA: enzyme linked immunosorbent assay
- FCS: foetal calf serum
- GST: glutathione S-transferase
- HEPES: 4-(2-hydroxyethyl)1-piperizineethanesulfonic acid
- HIV: human immunodeficiency virus
- HRP: horseradish peroxidase
- IFA: incomplete Freund’s adjuvant
- IFN-\(\gamma\): interferon gamma
- Ig: immunoglobulin
- IgE: immunoglobulin E
- IL-4: interleukin-4
- IL-5: interleukin-5
- IL-13: interleukin-13
- IPTG: isopropyl-\(\beta\)-D-thiogalactopyroniside
- LB: Luria broth
- mAb: monoclonal antibody
- min: minute
- mRNA: messenger ribonucleic acid
- NPC2: Neimann-Pick Type C2
- nDer p 1: natural Der p 1
- nDer p 2: natural Der p 2
- pIII: protein III
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<td>protein VIII</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>passive cutaneous anaphylaxis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PSB</td>
<td>phage storage buffer</td>
</tr>
<tr>
<td>rDer p 2</td>
<td>recombinant Der p 2</td>
</tr>
<tr>
<td>RAST</td>
<td>radioallergosorbent test</td>
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<tr>
<td>Rh</td>
<td>Rhesus factor</td>
</tr>
<tr>
<td>RF</td>
<td>replicative form</td>
</tr>
<tr>
<td>Rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SIT</td>
<td>specific immunotherapy</td>
</tr>
<tr>
<td>SMM</td>
<td>spent mite medium</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>Tₘ</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethyl benzidine</td>
</tr>
<tr>
<td>TRF</td>
<td>time resolved fluorescence</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indoyl-β-D-galactoside</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
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CHAPTER ONE

LITERATURE REVIEW
1.1 ASTHMA AND ALLERGY

Over the last 40 years of the twentieth century the prevalence and severity of asthma and allergic disease increased dramatically. Many studies have attempted to elucidate the causes of the increase, investigating factors such as air pollution, childhood immunisation, increased antibiotic use and dietary changes (Crater and Platts-Mills, 1998). Several studies suggested that the increase in asthma was part of an overall increase in skin test reactivity to environmental allergens; sensitisation to environmental allergens is the only identified risk factor for the development of asthma (Platts-Mills et al., 2000). At the turn of the century, the scale of the problem was such that it was estimated that over half of the population of the Western world were sensitised to one or more environmental allergens (Holgate, 1999).

The current treatments for allergic diseases such as rhinitis, dermatitis and asthma include allergen avoidance strategies as well as the use of corticosteroids and mediator antagonists such as antihistamines and antileukotrienes. Pharmacotherapies carry considerable expense as well as the risk of side effects. None are a cure for allergic diseases, only treating the end symptoms. Immunotherapy aims to actually prevent the development of allergic disease, as well as to ameliorate existing allergic disease. Current immunotherapy protocols have had some success, but they carry the risk of serious side effects as well as the possibility of developing new allergies (Allergen immunotherapy: a practice parameter, 2003). Immunotherapy protocols in use are also time consuming and expensive.

This thesis aims to utilise phage display libraries and monoclonal antibodies in order to isolate peptide mimotopes of allergens. These mimotopes will be explored for their utility as immunotherapeutic agents, as well as being used to examine the allergic response itself. The studies to be reviewed below demonstrate that mimotopes of allergens can be isolated from phage display libraries, and can be used for immunotherapy as well as reagents for the study of allergic reactions.
1.1.1 Asthma

Allergic asthma is a multi-step process (reviewed by Ferreira (2004)), consisting of a process of allergic sensitisation followed by tissue damage and repair. It develops in individuals who display an increased risk of Th2 allergic sensitisation and/or an increased susceptibility to lung epithelial injury and/or impaired healing of the lung epithelium. During normal conditions, irritants such as viruses or bacteria can cause damage to the lung epithelium which is then rapidly repaired by normal lung repair mechanisms. In the asthmatic individual, a functional disturbance of these repair mechanisms prevents this action, leading to permanent inflammation and eventual physical remodelling of the lungs.

1.1.1.1 Th2 Allergic Sensitisation

Sensitisation to otherwise innocuous environmental antigens, or allergens, is the strongest risk factor for the development of asthma (Platts-Mills et al., 2000). Initial sensitisation to allergens occurs at the mucosa, and involves a complex interplay of inflammatory cells (Lemanske and Busse, 1997). Antigen presenting cells (APCs) such as dendritic cells (DCs) take up allergens, process them and present them to naïve (Th0) CD4+ T cells in the peripheral lymphoid organs (Ferreira, 2004). These T cells are activated by this interaction with APCs, with some acquiring a Th2 phenotype, preferentially producing interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13).

One of the major actions of these cytokines is the activation of B cells, inducing an isotype switch from IgM to IgE production. IgE produced by the activated B cells binds to mast cells located below the lung epithelium via the high affinity receptor FceRI, sensitising them. IgE also interacts with a low affinity receptor, CD23 (or FeγRII) on B cells, enhancing B cell immune responses such as antigen presentation and the amplification of IgE production (Broide, 2001). IgE also has the effect of upregulating the production of its receptors; conversely, studies have shown that reducing the level of IgE in the serum reduces the expression of IgE specific receptors (Oettgen and Geha, 2001).
1.1.1.2 The Asthmatic Response

The asthmatic response induced by allergen is biphasic, consisting of early and late phase responses (Ferreira, 2004). The early phase response occurs within minutes of allergen exposure, and is primarily a response triggered by the cross-linking of mast cell-bound IgE. This interaction triggers mast cell degranulation, releasing mediators such as histamine, tryptase, leukotrienes and prostaglandins. Together, these mediators act to bring about contraction of airway smooth muscle, vasodilation and an increase in mucous secretion; these factors combine to result in narrowing of the airway, or bronchoconstriction.

The late phase asthmatic response is an inflammatory response, occurring within hours of allergen exposure. It is a mixed response, probably mediated in part by a cytokine/chemokine cascade and migration of inflammatory cells and in part by presentation of allergen by mature dendritic cells, which present the allergen in turn to memory T cells within the lung mucosa and peripheral lymphoid organs. These cells release further chemotactic factors which induce an influx of basophils, eosinophils, macrophages and platelets into the bronchus. Airway eosinophilia in particular is characteristic of asthma. The infiltrating cells produce inflammatory molecules, which result in chronic bronchial hyperreactivity, both to allergens and other factors such as methacholine and cold air (Lemanske and Busse, 1997). The inflammation also gives rise to airway remodelling involving thickening of the airway wall, increased collagen deposition and increased vascularity as well as hypertrophy and hyperplasia of airway smooth muscle (Davies et al., 2003; Holt et al., 1999).

1.1.2 Allergen Immunotherapy

Specific allergen immunotherapy (SIT) is the process of administration of allergen extracts to an individual with the intent of modifying or abolishing the symptoms associated with atopic allergy. Immunotherapy was first described in 1911 (Noon, 1911), when increasing doses of allergen were administered subcutaneously to patients in order to treat hayfever. Many modern immunotherapeutic strategies vary little from this original procedure.
Immunotherapy has been successful in the treatment of diseases such as allergic rhinitis mediated by grass, ragweed and birch pollens, and has been the standard method of choice for the treatment of Hymenoptera sting allergy since 1978 (Hunt et al., 1978). A subset of asthmatic patients also benefit from allergen immunotherapy (Abramson et al., 1995).

1.1.2.1 Mechanisms of Immunotherapy

The precise mechanisms behind successful immunotherapy are unknown, but are thought to include the generation of blocking antibodies, a reduction in specific IgE in the long term, reduced recruitment of effector cells, altered cytokine balance, T cell anergy and induction of regulatory T cells (Frew, 2003).

Allergen-specific IgE levels initially rise in the early stages of SIT, but fall back to pretreatment levels once the patient has entered the maintenance stage. Specific IgG levels also rise initially, with IgG1 peaking within 2-3 months and IgG4 peaking after two years. For many patients, there is an inverse correlation between IgG levels and cutaneous late-phase reactions. Furthermore, the usual seasonal rise of IgE in response to increased levels of allergen in the environment is not seen, leading overall to a reduced level of IgE in the long term (Weber, 1997).

Other changes seen in successful SIT include decreased T cell allergen-specific proliferation, reduced ratio of IL-4, IL-5 or IL-13 production relative to IFN-γ, decreased expression of IL-4 and IL-5 mRNA and decreased numbers of mast cells, basophils and eosinophils in the lungs (Lewis, 2002).

While immunotherapy has been successful for the treatment of some allergic disease, the treatment itself carries the risk of anaphylaxis, as well as the potential for the development of new IgE responses to any other allergens present in the extract used (Ball et al., 1999b). Improvement in the characterisation and standardisation of allergens has allowed for safer and more predictable treatments, with lower risks of anaphylaxis (Weber, 1997), however, the treatments themselves are protracted and
expensive, and are not always effective, indicating the need for improved immunotherapeutic regimes.

### 1.1.2.2 B Cell Epitopes as Immunotherapeutic Agents

One approach to improving immunotherapy has been the development of novel therapeutic agents in the form of B and T cell epitopes of allergens. It is envisaged that peptides representing these epitopes could be used in order to induce blocking antibodies in the case of B cell epitopes, and tolerance or immunoregulation in the case of T cell epitopes. The major advantage to the use of peptides is the fact that they are extremely unlikely to be able to cross-link mast cell-bound IgE and lead to an anaphylactic reaction. (Lewis, 2002).

Peptides representing B cell epitopes could be utilised as immunotherapeutic agents in two different ways. First, the peptides could be locally applied to the mucosa, where they would saturate mast cell-bound IgE, thus preventing allergen binding and cross-linking. Secondly, the peptides could also be used as active immunisation agents, inducing blocking IgG antibodies which would bind to the allergen, preventing it from binding to mast cell-bound IgE (Valenta et al., 1998) or modulating other aspects of the allergen response.

B cell epitopes are often considered to exist in one of two forms. Continuous epitopes are represented by an unbroken stretch of amino acids in the primary protein sequence, while conformational epitopes are formed by folding of the protein, bringing distant residues together where they can be bound by antibodies as a unit. The mapping of continuous epitopes has been achieved by screening for antibody binding to overlapping peptides representing the entire protein sequence. Conformational epitopes have traditionally been much more difficult to map, given the fact that they are not represented in the protein primary sequence. While continuous portions of conformational epitopes can be identified via the use of overlapping peptides, mapping of the whole epitope has only been achieved by the use of X-ray crystallography. Due to the absolute requirement of purified antigen-antibody complexes for this procedure, it is not always a feasible process.
Several studies have investigated the antigen-antibody interaction. The interaction of hen egg white lysozyme with monoclonal antibodies has served as a model system. Three antibodies have been identified which are specific for the same epitope on lysozyme, though their affinities vary. Modelling of the interaction between these antibodies and lysozyme revealed that they recognise some of the same residues, but different atoms of these residues. This allows two antibodies to bind at the same time to the same epitope without hindering each other (Mohan et al., 2003).

The B-cell epitopes of Bet v 1 were studied by Mirza et al (2000), who postulated that epitopes of allergens would be more likely to be found within areas conserved between homologous proteins from different species. As individuals are exposed more frequently to regions conserved between a wide range of allergens, they would be more likely to raise an antibody response to these regions. The three-dimensional structure of Bet v 1 complexed to a murine mAb was resolved; the antibody bound to a conserved area of the protein previously demonstrated to be antigenic. The epitope was conformational, but 80% of the residues involved were part of two separate continuous stretches of amino acids.

Gelatine has also been utilised as a model system for the investigation of protein epitopes. Due to its lack of a rigid structure, the protein itself is poorly antigenic. When gelatine was conjugated to aromatic amino acids (tyrosine, tryptophan and phenylalanine), the resultant protein became antigenic, leading to the conclusion that the aromatic amino acids were likely to be important in antigenic determinants, probably because their shape and size forced the protein into a rigid conformation (Sela and Arnon, 1960).

While theoretically any portion of a protein can potentially be bound by an antibody, experimental evidence suggests that proteins possess a few prominent B cell epitopes representing conserved immunodominant structures. An analysis of the birch allergen Bet v 1 demonstrated that B cell epitopes mapped to surface exposed areas which were conserved across species (Gajhede et al., 1996). It has also been observed that antibody binding regions of proteins frequently contain a high proportion of charged and polar residues, and are often located on flexible portions of the protein, such as hydrophilic loops (Hopp, 1986).
1.1.3 Blocking Antibodies

It has long been recognised that successful immunotherapy results in the generation of allergen-specific IgG antibodies (reviewed in Flicker and Valenta, 2003). These antibodies were originally termed “blocking antibodies” due to their ability to inhibit immediate skin reactions to allergens.

Blocking antibodies were originally thought to be of the IgG4 isotype in humans (Aalberse et al., 1983); the equivalent in mice is the IgG1 isotype (Vrtala et al., 1998). IgG4 was proposed as a candidate for blocking antibody isotype for several reasons. Immunotherapy results in increased levels of allergen-specific IgG4, an isotype of antibody that does not activate complement or cross-link cell-bound antigen, and has a demonstrated ability to block allergen-induced IgE-dependant histamine release from basophils (Garcia et al., 1993). IgG4, in combination with other allergen-specific immunoglobulins, also plays a role in the suppression of allergen-specific T cell and antibody-mediated enhancement of the immune response (van Neerven et al., 1999). IgG4 antibodies are monovalent, and therefore unlikely to be able to cross-link allergen bound to IgE or mast cells (Schuurman et al., 1999; van der Zee et al., 1986).

The role of allergen-specific IgG4 as a blocking antibody has been a controversial issue. While it is acknowledged that successful immunotherapy results in high levels of allergen-specific IgG1 and IgG4, studies have generated conflicting data as to the specific role of IgG4 in blocking the allergic response. A retrospective survey of immunotherapy trials in 1987 concluded that high levels of allergen-specific IgG4 at the end of therapy were in fact associated with failure of immunotherapy, and therefore this isotype could not be acting in a blocking fashion (Djurup and Malling, 1987). In 2003, it was proposed that these immunotherapy trials studied to reach this conclusion were in fact flawed, as they used allergen extracts rather than purified allergens to quantitate antibody levels in serum. The use of extracts means that IgG levels against non-allergenic components in the extract was measured along with the allergen-specific IgG (Flicker and Valenta, 2003).

A series of more recent studies have reaffirmed the role of blocking antibodies in immunotherapy, concluding that successful immunotherapy does indeed result in the
generation of allergen-specific IgG4 and as well as IgG1 (Ball et al., 1999a; Ejrnæs et al., 2004).

The role of blocking antibodies in grass pollen immunotherapy was studied by Nouri-Aria et al. (2004). Successful immunotherapy resulted in the generation of a 60 to 80 fold increase in allergen specific IgG, and a 100 fold increase in allergen specific IgG4. Further, post-immunotherapy serum was capable of blocking the binding of allergen-IgE complexes to B cells, an activity that co-eluted with the IgG4 fraction of the serum. Increases in serum IgG and blocking activity of the serum correlated with clinical improvement.

Correlation of clinical improvement of patients with the generation of both allergen-specific IgG1 and IgG4 was also seen in another study of grass pollen immunotherapy. Allergen-specific IgG2 and IgM were also observed in several individuals. Post-immunotherapy serum was capable of inhibiting basophil histamine release; those patients whose serum demonstrated blocking activity also displayed a blunting in seasonal IgE increases (Mothes et al., 2003), indicating successful immunotherapy.

The interaction of blocking antibodies with T cells has also been studied. Serum from patients who had undergone grass pollen immunotherapy has been shown to block the binding of allergen-IgE complexes to B cells, as well as subsequent allergen presentation to T cells (Wachholz et al., 2003). The blocking antibodies disrupted the formation of the allergen-IgE complex by binding to the allergen, rather than binding directly to the B cells. A similar study looked at the serum from patients who had undergone birch pollen immunotherapy. Post-immunotherapy serum was able to inhibit serum-facilitated allergen presentation, and thus the activation of T cells. This activity was isolated to the IgG fraction of the serum (van Neerven et al., 1999).

Blocking antibodies have also been studied in a murine model. IgG1 antibodies generated from immunisation of mice with both natural and recombinant timothy grass pollen allergens are able to inhibit the human IgE-allergen interaction, as well as allergen-induced human basophil degranulation (Vrtala et al., 1998). In this case, the murine IgG1 antibodies were directed against the same epitope on the allergen as the human IgE.
The generation of IgG subclass blocking antibodies is dependent on the allergen dose used in immunotherapy. Kolbe et al. (1995) immunised CBA/J mice with bee venom phospholipase A\textsubscript{2} and keyhole limpet haemocyanin. Low doses of antigen induced high levels of both IgE and IgG, while high doses induced high levels of IgG but only low levels of IgE. When small doses of antigen were used, it was observed that the IgG and IgE generated were directed against distinct and non-overlapping epitopes, meaning that the IgG antibodies would not be able to block the IgE-antigen interaction. It was only when large doses of antigen were used that blocking IgG antibodies were produced, recognising the same epitope as IgE.

It can be concluded from the above studies that the generation of blocking antibodies is indeed involved in the success of immunotherapy, and may be one of the central mechanisms of immunotherapy. In mice the blocking antibodies are of the IgG1 isotype, while in humans they may be any of the IgG isotypes, though more commonly IgG4. These blocking antibodies displace IgE from the allergen-IgE complex, and therefore must be directed against the same epitope and have the same or higher affinity for the allergen as IgE.

### 1.1.3.1 Antibodies as Regulators

Antibodies can act to enhance or repress immune responses to antigens (reviewed in Heyman, 2000). The effect that an antibody has depends on both its class, specificity and the time of delivery relative to the introduction of antigen. To enhance an immune response, the antibody must be present at the same time as the antigen, or delivered within several hours of antigen challenge. Suppression by immunoglobulins has been demonstrated to be the most effective when the antibody is delivered within 24hr of antigen challenge.

Suppression of immune responses by IgG has been demonstrated in the murine reaction to erythrocytes. The suppression was dose dependent, and independent of antibody subclass. The suppressive ability of an antibody correlates with antibody affinity. The classic example of antibody-mediated suppression is the suppression of responses to erythrocytes, utilised clinically in the prevention of Rh- women from becoming immunised to foetal Rh+ erythrocytes (Karlsson et al., 1999). The mechanism for antibody-mediated suppression is unknown, but thought to possibly include the
following: antibodies could bind to antigenic epitopes, masking them from B cells, antibody antigen complexes could be phagocytosed before they could activate B cells, or the antigen antibody complex could act to cross-link the B cell receptor and FcγRIIB, an inhibitory receptor expressed on B cells.

The effects of administering IgG to sensitised mice has been investigated (Sehra et al., 2003). Mice sensitised to ovalbumin were administered anti-ovalbumin IgG via the intranasal route, and then challenged with ovalbumin. Groups of mice who had received the antibody displayed reduced eosinophilic inflammation and goblet cell metaplasia in the lungs, and demonstrated a skewing of the immune response towards a Th1 response. Responses to bystander allergens were also repressed. The suppression was independent of isotype, both IgG1 and IgG2a antibodies induced suppression. The effect was completely dependent on the development of specific antibody-antigen complexes.

Enhancement of immune reactions by IgM, IgG and IgE is antigen specific, but not epitope specific, i.e. an antibody specific for one epitope of an antigen will increase reactivity to all of the epitopes of that protein. Enhancement does not appear to correlate with affinity or isotype; IgG1, IgG2a and IgG3 are all capable of enhancing immune reactions in mice. The enhancement is mediated by FcγRs located on murine macrophages, monocytes and neutrophils (Ravetch and Bolland, 2001).

There are three distinct classes of FcγRs, differing in molecular size, cellular distribution, function and affinity to IgG isotypes. In the mouse, FcγRI and FcγRIII are activating receptors. FcγRI is mainly expressed on macrophages and binds IgG (IgG2a and IgG2b, but not IgG1) with high affinity. FcγRIII is a low affinity receptor, binding complexed IgG1, IgG2a and IgG2b but not IgG3. FcγRII is an inhibitory receptor, co-expressed with FcγRIII on myeloid cells including neutrophils, mast cells and macrophages (Baumann et al., 2002). Allergen-specific anaphylactic IgG1 has been implicated as a possible activator of the allergic reaction, binding to FcγRIII on murine mast cells, activating the cells (Macedo-Soares et al., 2004).
1.2 MAJOR HOUSE DUST MITE ALLERGENS

The allergens most frequently encountered in allergic disease are ubiquitous antigens that are generally harmless, but to which an inappropriate immune response are made by atopic individuals. In order to be classified as an allergen, an antigen must be capable of inducing the production of high-affinity IgE as well as an allergic reaction. A major allergen for any species is defined on the prevalence of IgE in allergic patients, inducing more than 50% of the total allergenic reactivity in more than 20% of patients (Aalberse, 2000). While allergens can be classified into a small number of structural classes, there are no individual structural features that are known to be common for all allergens.

One of the major sources of environmental indoor allergens is the house dust mite. Three species of house dust mite dominate in most species of the world, *Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphus maynei*. Of these, *D. pteronyssinus* is the most common house dust mite in Australia. Allergy to house dust mites is the only risk factor that has been linked to the development of asthma. There are currently at least 19 known allergens produced by house dust mites (Thomas et al., 2002); *D. pteronyssinus* produces two major allergens, Der p 1 and Der p 2.

1.2.2 Der p 1

The first major allergen to be identified from *D. pteronyssinus* was Der p 1, a 25kDa cysteine protease (Chapman and Platts-Mills, 1980). The cDNA encoding the protein was sequenced and shown to demonstrate homology to papain and other cysteine proteases (Chua et al., 1988; Platts-Mills, 1993). Der p 1 binds IgE at high affinity, with 70-90% of house dust mite allergic patients producing Der p 1-specific antibodies (O'Brien and Thomas, 1994). It is present in house dust mite extracts at a concentration of 10-100μg/ml, particularly localising to mite faecal pellets (Chapman et al., 1987).

Murine monoclonal antibodies raised against Der p 1 are species specific; they often do not bind well to the Der f 1 allergen from *D. farinae*, despite a 70-80% sequence homology between Der p 1 and Der f 1. In contrast, human IgG and IgE antibodies to
group one allergens are often highly cross-reactive between Der p 1 and Der f 1 (Baldo and Donovan, 1990; Chapman et al., 1987; Lind et al., 1988).

A three-dimensional model of Der p 1 has have been generated by comparison with the known structures of related cysteine proteases (Topham et al., 1994). The protein folds into two domains, with the active site of the enzyme located in a groove between the two domains. This structure has been validated by the use of circular dichroism analysis (Takai et al., 2005) and more recently, the crystal structure of recombinant pro-Der p 1 has been solved (Meno et al., 2005).

Regions of Der p 1 recognised by human T cells have been mapped. The linear sequence of residues 107-131 represents a region of overlapping T cell epitopes (Hales and Thomas, 1997; O'Brien et al., 1994; Yssel et al., 1992). Regions of the molecule bound by antibodies have also been mapped, including murine monoclonal antibodies (Chapman et al., 1984; Greene et al., 1990; Lind et al., 1988), human IgE (Greene et al., 1991; Greene and Thomas, 1992; Heymann et al., 1986) and human IgG (Duchateau et al., 1997). All of the known epitopes of Der p 1 are conformational and located on the surface of the three-dimensional model of the protein. Of particular interest is the work of Greene and Thomas (1992), which identified the residues 101-111 as being part of an IgE binding structure. This sequence is located on a flexible loop and overlaps with the region of overlapping T cell epitopes.

Murine monoclonal antibodies raised in BALB/c mice against Der p 1 have been classified into four groups on the basis of competition assays (Lind et al., 1988), representing four antibody binding regions on the surface of the protein. Some of the murine antibodies are capable of inhibiting the binding of human IgE to Der p 1, though large excesses of the competing mAb are required. The mAb 4C1, which is cross reactive with both Der p 1 and Der f 1, is capable of partially inhibiting human IgE binding to Der p 1 (Chapman et al., 1987). The epitope on Der p 1 recognised by 4C1 was mapped to the region 60-80, a highly conserved region between Der p 1 and Der f 1 (Greene, unpublished data). This region, represented by a loop structure connecting the two domains of Der p 1, is also the major site recognised by rabbit antiserum (Chapman et al., 1987), and has been theorised to be a major epitope recognised by humans, mice and rabbits.
The major mite allergens show frequent variation of sequence attributed to allelic polymorphism. Polymorphisms of Der p 1 have been observed in both cultured and wild mites; these include A 3 G, H 50 Y, H 72 S, E 81 K, A 124 V, S 136 T and E 215 Q, all of which are surface-exposed residues (Robinson et al., 1997). They have not been examined for their effect on antigenicity because the production of correctly folded recombinant Der p 1, which would be required for this analysis, has been difficult.

1.2.3 Der p 2

The second major allergen of *D. pteronyssinus* is Der p 2. Eighty percent of allergic patients produce Der p 2 specific IgE (O'Brien and Thomas, 1994). The precise function of the protein in the mite is unknown, but the protein displays homology to a family of epididymal proteins (Thomas et al., 2002), although the allergen is found in both male and female mites (Park et al., 2000). The primary sequence of the protein also displays homology to the mammalian secretory protein HE1 and the Neimann-Pick type C2 (NPC2) protein. The NPC2 protein binds cholesterol with high affinity; mutations are associated with NPC2 disease, a fatal disorder characterised by defective cholesterol transport from lysozymes (Gruber et al., 2004). The three-dimensional structure of Der p 2 has been resolved by X-ray crystallography (Derewenda et al., 2002). The protein is an immunoglobulin-like protein consisting mainly of β-sheets. There are three disulphide bonds formed between cysteines 21 and 27, 73 and 78, and 8 and 119. The allergen, as well as the NPC2/HE1 homologs, possesses two highly conserved basic clusters of residues which form a hydrophobic pocket in the folded protein. This homology suggests that Der p 2 is involved with the binding of an as-yet unidentified hydrophobic ligand.

The epitopes of Der p 2 recognised by antibodies and T cells have been studied via various methodologies. Hakkaart et al. (1998) utilised site-directed mutagenesis to map epitopes recognised by murine anti-Der p 2 monoclonal antibodies. The disulphide bonds were necessary for antibody binding, indicating that the epitopes are conformational. The antibodies could be classified into three groups, each group recognising a separate antigenic region on Der p 2. Binding of the mAb DpX is dependent on the stretch of amino acids from 65-80, while IgE binding decreased when
the regions 45-48, 67-69 and 89-90 are mutated, indicating that these areas are involved in IgE epitopes.

Mueller et al. (2001) further defined the murine mAb epitopes on Der p 2 by the use of alanine scanning and hydrogen exchange nuclear magnetic resonance spectroscopy. One mAb from each of the three groups defined by Hakkaart et al. (1998) was investigated. The residues binding each of the mAbs are listed in Table 1.1, and shown diagrammatically on the three-dimensional model of Der p 2 in Figure 1.1.

<table>
<thead>
<tr>
<th>mAb</th>
<th>Amino acid residues within predicted epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>7A1</td>
<td>18-20, 27-37, 55-63, 92-106, 123-129</td>
</tr>
<tr>
<td>DpX</td>
<td>47-52, 68-81, 84-91, 110-112</td>
</tr>
<tr>
<td>6D6</td>
<td>7-13, 41-55, 70-73, 76, 80, 107-120</td>
</tr>
</tbody>
</table>

**Table 1.1** Der p 2 Residues involved in murine monoclonal antibody epitopes
(from Mueller et al., 2001)
Investigation of the epitopes recognised by human IgE on Der p 2 has been undertaken via the use of competition assays between allergic patient IgE and murine mAbs directed against Der p 2 (Mueller et al., 2001). Inhibition patterns varied on an individual basis, with each patient serum being inhibited by a unique group of mAbs. In some cases, the IgE response was clearly monovalent, with the majority of IgE binding inhibited by a single mAb. Overall, most of the patient IgE was inhibited by the mAbs DpX and 6D6, indicating that the epitopes recognised by these mAbs are in the same region as the human IgE epitopes.
The epitopes of Der p 2 recognised by human T cells have been mapped, with one region (residues 21-35) containing the immunodominant epitope across mouse species (Wu et al., 2002). Human T cells frequently recognise epitopes in the region 105-129, though T cells can recognise any part of the Der p 2 molecule (O’Hehir et al., 1993).

Several different isoforms of Der p 2 have been isolated from cultured and wild mites; these vary mainly at amino acid positions 40, 47, 111 and 114 (Smith et al., 2001). Of these residues, 47 and 114 are exposed to solvent. There is evidence that polymorphisms at these residues can affect murine mAb binding (Smith et al., 2001) as well as IgE binding (Hales et al., 2002; Park et al., 2002). The polymorphisms of several Der p 2 isoforms are shown in Table 1.2.

<table>
<thead>
<tr>
<th>Residue</th>
<th>40</th>
<th>47</th>
<th>98</th>
<th>111</th>
<th>114</th>
<th>116</th>
</tr>
</thead>
<tbody>
<tr>
<td>0101</td>
<td>V</td>
<td>T</td>
<td>A</td>
<td>M</td>
<td>D</td>
<td>V</td>
</tr>
<tr>
<td>0102</td>
<td>L</td>
<td>S</td>
<td>A</td>
<td>L</td>
<td>N</td>
<td>V</td>
</tr>
<tr>
<td>0107</td>
<td>V</td>
<td>S</td>
<td>A</td>
<td>M</td>
<td>D</td>
<td>V</td>
</tr>
<tr>
<td>0108</td>
<td>V</td>
<td>S</td>
<td>A</td>
<td>M</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

**Table 1.2 Polymorphisms of Der p 2 from Perth environmental mites**
(from Hales et al., 2002). The residue number is shown across the top of the table, with the amino acids at each of these sites for each form of Der p 2 (0101, 0102, 0107 and 0108) shown per line.

Studies have shown that Der p 2 is highly immunogenic when delivered via the intraperitoneal route in different mouse strains (C57BL/6, C3H, CBA, A/J, AKR, BALB.B, BALB.K, BALB.A10 and BALB.BD (2R)). BALB/c mice produce only a weak antibody response, indicating that mice of the haplotype H-2^d^ do not respond well to Der p 2 (Ovsyannikova et al., 1994). The T cell response of different mouse strains to Der p 2 has also been examined (Hoyne et al., 1993) with a similar pattern to the antibody response seen. Mice of haplotype H-2^b^ were high responders, H-2^k^ were intermediate responders and H-2^d^ were low responders to Der p 2.
1.3 MODEL ALLERGENS AND EPITOPES USED IN THIS STUDY

1.3.1 Papain

Papain is a cysteine protease derived from papaya latex which is used industrially as a meat tenderiser and beer clarification agent as well as in many pharmaceutical-type products. Human exposure to papain has been associated with hypersensitivity reactions (Baur et al., 1982; Zentner et al., 1997), and has been demonstrated to induce an IgG1 (Th2) response in mice (Chambers et al., 1998).

The structure of papain has been solved by X-ray crystallography (Drenth et al., 1968; Kamphus et al., 1984; Yamamoto et al., 1991). The protein displays homology to Der p 1 and has been theorised to share at least one cross-reactive IgE epitope with the house dust mite allergen (Furmonaviciene et al., 2000), although this has not been demonstrated; these two proteins only share 25% amino acid similarity.

1.3.2 The FLAG Epitope

The FLAG peptide (DYKDDDDK) is a widely used affinity tag. The peptide is hydrophilic and immunogenic (Hopp et al., 1988). Three antibodies directed against the FLAG peptide are commercially available, designated M1, M2 and M5. These antibodies vary in their recognition of FLAG relative to the position of the tag within the fusion protein as well as their requirement for calcium for binding. The anti-FLAG M2 mAb recognises the FLAG peptide at both amino and carboxyl termini as well as internally and has no calcium requirement (Einhauer and Jungbauer, 2001).

The FLAG peptide is often used as a fusion tag for the isolation of recombinant proteins for several reasons. The addition of the FLAG peptide usually does not interfere with the structural and functional properties of proteins and is able to be cleaved from the fusion partner by the use of enterokinase. There is no known cross-reactivity of FLAG with *E. coli* proteins present in a crude extract. The peptide is stable at the amino
terminus of a fusion protein, and is not cleaved by any *E. coli* proteases (Knappik and Pluckthun, 1994).

The residues of the FLAG peptide critical for mAb binding have been defined by alanine scanning. For the M2 mAb, residues Y2 and K3, as well as D1 and D6 to a lesser extent, are the critical residues. The antigenicity of peptides mutated at residues other than these has not been investigated (Miceli et al., 1994).

**1.4 PHAGE DISPLAY**

Phage display libraries consist of tens to hundreds of millions of short, variable peptides displayed on the surface of bacteriophage virions (Scott, 1992). These libraries are a rich source of ligands, and have been used to identify and isolate binding partners of many different molecules.

The length of the displayed peptide is critical in the determination of the diversity of the library, and therefore its use in the isolation of ligands. In order for a library to cover every possible permutation of a random heptapeptide, for example, the library would need to contain $1.3 \times 10^9$ unique sequences. As the length of the displayed peptide increases, the number of unique sequences in the library required for complete coverage increases exponentially. This means that library size also increases exponentially, which can become more difficult to achieve due to space constraints (Burritt et al., 1996). Generally, the smaller peptide libraries, displaying hexapeptides or heptapeptides, have complete coverage, while larger peptide libraries, for example displaying dodecapeptides, do not.

Phage display libraries have been exploited for many different purposes, including the mapping of protein-protein interactions. It has been observed that the primary structures of peptides binding to the interaction partner often resemble the primary sequence of the original target protein, a phenomenon that Kay et al. (2000) termed “convergent evolution”. This, however, is not always the case in that peptides can produce shapes that are similar to the target, rather than a similar sequence.
Phage display libraries can also be used to isolate antagonists or agonists of protein interactions, identify unknown ligands for proteins and identify lead peptides for drug design, among many other uses (Kay et al., 2001).

Several different vehicles have been employed for the display of libraries of peptides, including bacterial flagella, λ phage, T4 phage, T7 phage and filamentous bacteriophage. Filamentous phage vectors were the first developed, and have been the most widely utilised (Rodi and Makowski, 1999; Parmley and Smith, 1988; Smith, 1985; Smith and Scott, 1993).

1.4.1 Filamentous Phage Display

The use of the filamentous bacteriophage as a vehicle for the display of peptides was pioneered by Smith (1985), who demonstrated that it was possible to insert a foreign sequence into the phage virion, resulting in the expression of the foreign protein on the phage surface as a fusion with a phage coat protein. This foreign protein could then be utilised to isolate the recombinant bacteriophage by a process of affinity selection. It has since been shown that foreign DNA of up to 12,000 nucleotides in length can be cloned into the wild type filamentous phage genome, which is 5000-8000 nucleotides in length, without affecting the viability of the recombinant phage (Marvin, 1998).

The filamentous bacteriophages consist of a group of male-specific non-tailed viruses, including the well-studied fl, fd and M13 phages. All of these phages, collectively known as the Ff phages, infect *E. coli* via adsorption to the F pilus (Rasched and Oberer, 1986). All of the filamentous phages are physically simple, consisting of just five coat proteins enclosing a covalently closed ssDNA genome (Cabilly, 1999). The majority of the protein coat is composed of approximately 2800 copies of protein VIII (pVIII). At one end of the virion five copies each of protein III (pIII) and protein VI (pVI) are present in a cluster, while at the other end five copies each of protein VII (pVII) and protein IX (pIX) are present in another cluster (Smith, 1988). Figure 1.2 shows a schematic diagram of the filamentous phage virion.
Figure 1.2 Schematic diagram of filamentous phage structure.

The use of filamentous bacteriophages as expression vectors for library construction has several major advantages. First, the small size of the virion allows the construction of large libraries in small volumes, increasing the possible complexity that can be achieved. The phage virion itself links the physical properties of the displayed peptide with the genetic information about the peptide, allowing the peptide to be selected physically and immediately sequenced. The stability of the phage particle to 95°C and a pH as acidic as 2.0 facilitates the elution of selected phage from a target molecule by a simple pH change without affecting phage viability (Cesareni, 1992). Filamentous phage are lysogenic rather than lytic (infection slows phage growth enough for the development of turbid plaques on solid media), allowing the propagation of phage to high titres in cultures as well as the easy isolation of RF DNA and propagation of the phage as a plasmid (Smith, 1988).

Filamentous phage are well known to be immunogenic. Molenaar et al. (2002) immunised C57BL/6J mice with wild type M13 and studied the tissue distribution of the phage particles. The half-life of phage particles in the blood was 4.5hr, while phage particles could be demonstrated as localising to the lungs, liver and spleen within 30-90min of injection.

Two coat proteins have been used to display peptides, the minor coat protein pIII and the major coat protein pVIII. For display on both of these proteins, the foreign peptide is inserted at or close to the amino terminus (Scott, 1992). Both of these proteins can be used for multivalent or monovalent display, dependant on the vector employed.
1.4.1.1 Protein VIII Display

Protein VIII (pVIII) is the major coat protein of the filamentous phage, present at 2,800 copies per virion. The mature protein is 50 amino acids in length and highly hydrophobic, forming aggregates in aqueous solution (Rasched and Oberer, 1986). Foreign peptides are expressed as amino-terminal fusions with pVIII. Peptides over the length of five amino acids disrupt phage assembly; this limitation can be overcome by construction of a vector that expresses both recombinant and wild-type pVIII (Scott, 1992). These vectors express approximately 150 copies of recombinant pVIII; each recombinant protein must be surrounded by wild-type pVIII in order for phage assembly to be successful. The expression of both recombinant and wild-type pVIII allows the expression of larger proteins such as Fv (Scott, 1992).

1.4.1.2 Protein III Display

Protein III (pIII) is a 406 amino acid minor coat protein, present at five copies per virion grouped at one end of the phage particle. This protein facilitates adsorption of the phage to the host bacterial cell F pilus during infection (Rasched and Oberer, 1986). As for pVIII display, foreign peptides are displayed as amino-terminal fusions. Protein III has more flexibility in the length of foreign sequence that can be inserted without affecting phage assembly or infection; entire protein domains have been expressed in this fashion. As with pVIII display, vectors can be constructed that express both recombinant and wild-type pIII, resulting in monovalent display (Scott, 1992). The first vectors employing pIII were the fUSE vectors, based on the filamentous phage fd-tet (Parmley and Smith, 1988).

Examples of libraries that utilise pIII to display random peptides include the PhD series of libraries from New England Biolabs. These libraries are based on the filamentous bacteriophage M13KE and express random dodecamers (PhD12), heptamers (PhD7) or disulphide-constrained heptamers (PhDC7C). The PhDC7C library expresses random heptamers flanked by two cysteines, which are assumed to form a disulphide bond and this force the peptide into a fixed conformation, as opposed to non-constrained libraries, in which the peptides can presumably adopt several different conformations (Lowman,
1997). Restriction of the conformation of the peptide is thought to enhance the affinity of binding to its target (Schatz, 1994).

1.4.2 T7 Phage Display

An alternative vector for phage display is the lytic bacteriophage T7. The T7 virion consists of an icosahedral head in which a linear double stranded DNA genome is enclosed, and a short tail. The head of the phage is composed mainly of the major coat protein, protein 10 (p10) (Hausmann, 1988). p10 is naturally present as two variants, p10A and p10B (Gabashvii et al., 1997). It was this natural variation in p10 that led to the development of the T7Select vectors (Novagen). These vectors display foreign peptides or proteins as carboxyl-terminal fusions with the p10B protein, expressing between 1-415 copies of the protein or peptide per virion. The major advantages that T7 phage display has over filamentous phage display are a rapidity of growth, which facilitates speed of biopanning, and the fact that the T7 phage is lytic means that expressed proteins do not need to be capable of secretion through the bacterial cell membrane (Rosenberg et al., 1996).
Figure 1.3  The T7 Virion
(Image from Rosenberg et al., 1996). A cross section of part of the virion head is shown, with a single p10 subunit darkened.

T7 phage vectors have been used to construct random peptide libraries (Houshmand et al., 1999; Smith et al., 2002). The use of these vectors for immunisation has also been investigated. One group (Sokoloff et al., 2000) found that T7 phage were cleared rapidly from the serum in rats; the clearance was mediated by complement and natural antibodies and was specific to the peptides displayed by the phage. More recently, it has been shown that mice clear T7 phage from the blood rapidly in a B cell dependent manner; the presence of a displayed peptide did not alter clearance rate (Srivastava et al., 2004). The T7 phage display vectors have several advantages over the filamentous vectors. They are able to display large peptides, and these peptides are displayed as C-terminal fusions, making the transfer of peptides to other fusion vectors such as pGEX and pMAL simpler.

The T7 phage display vector has also been used to produce what has been described as a phylomer library (T.K. Heinrich et al., Telethon Institute for Child Health Research, personal communication; Watt and Thomas, AU patent #771534). This library was
constructed from random fragments of whole genomes from a series of divergent bacteria and cloned into the T7Select-415 phage display vector (Novagen). This library was envisaged to be a rich source of natural proteins domains which could be selected by biopanning with an appropriate protein or antibody. Natural peptide libraries have previously been used to isolate immunogenic peptides with success (Matthews et al., 2002).

1.4.3 Biopanning

Biopanning is the process of using affinity selection to isolate specific binding clones from a library. It can be performed in liquid phase, or more commonly, with the target protein adsorbed onto a solid surface. The process involves adding the phage library to the target, allowing it react, and then washing any unbound phage. Bound phage are eluted, titred, and then amplified by passage through an appropriate bacterial strain. This amplified eluate is then used as input into a further round of biopanning.

A successful round of biopanning will usually give a 1% yield of strongly binding phage. The first round of biopanning, where each clone may only be represented by a single virion, is therefore critical (Smith and Scott, 1993), and a large amount of target protein will often be used to maximise yield.

1.5 MIMOTOPES

The number of residues critical for the binding of any two proteins is small (three to ten residues) (Kay et al., 1998). In the case of monoclonal antibodies, the epitopes on the target protein may be either continuous, consisting of a stretch of 4-8 adjacent amino acids, or conformational, consisting of residues that are brought together by protein folding (Yip and Ward, 1999). Phage display libraries can be used both for epitope mapping, and for the isolation and identification of mimotopes, peptides which are able to mimic peptides both structurally and immunologically.
It has been previous observed that phage-displayed peptides can usually be found to mimic peptides, linear epitopes on folded proteins and carbohydrates. Mimics of conformational epitopes are found much more rarely (Bonnycastle et al., 1996)

A mimotope is a peptide which is capable of mimicking an antibody epitope. The term was first coined by Geysen et al. (1986), who defined a mimotope as “a molecule able to bind to the antigen-combining site of an antibody molecule, not necessarily identical with the epitope inducing the antibody, but an acceptable mimic of the essential features of the epitope”.

Mimotopes have been isolated for a number of antigens, including proteins and peptides as well as DNA (Sun et al., 2001) and polysaccharides (Harris et al., 2002; Pincus et al., 1998). Some of the studies which have isolated immunogenic mimotopes included targets such as human immunodeficiency virus (HIV) (Hewer and Meyer, 2003), simian HIV (Chen et al., 2001), respiratory syncytial virus (RSV) (Steward, 2001) and hepatitis C (Roccasecca et al., 2001)

1.5.1 Assessment of Mimotopes

The relevance of peptides selected from phage display libraries must be assessed, first by sequencing and comparison to the target protein sequence. The sequences do not necessarily need to have similarity; the similarity may exist in the shape of the binding domain. The peptides can also be assessed in in vitro studies, by competition with the native ligand of the target protein to determine specificity, and by affinity assays. Affinity of phage-displayed peptides can be problematic, because polyvalent phage can display avidity affects which can make it difficult to determine affinity (Lowman, 1997).

For a peptide to be classified as an immunogenic mimotope, it must be capable of mimicking the full protein immunologically, i.e. it must be capable of raising antibodies that cross-react against the original protein. This can be tested with phage-displayed mimotopes, as a free peptide, or cloned into another expression system to produce a fusion protein. Isolated peptide mimotopes would not be expected to be immunogenic, as they simply represent B cell epitopes, and are not able to attract T cell help necessary
for antibody production. The conjugation to a vehicle containing T helper cell epitopes means that the mimotope-vehicle would then be able to recruit bystander T cells to induce isotype switch and affinity maturation of B cells.

1.5.2 Allergen Mimotopes

Mimotopes of allergens are of particular interest because of their potential application to the elucidation of the allergic response on a molecular level as well as potential use as specific immunotherapeutic agents. One of the critical events in the allergic response is the cross-linking of mast cell-bound IgE by an allergen, which therefore must be multivalent (Turner and Kinet, 1999). Mimotopes should essentially act as haptens containing only one IgE epitope. As such they would be unlikely to be able to cross-link IgE and therefore constitute promising candidates for immunotherapy (Riemer et al., 2004).

The application of mimotopes to the treatment of allergy is two-fold. The peptides could be used for active immunotherapy, wherein blocking antibodies are generated which interfere with the allergen-IgE interaction. Mimotopes could also be used for passive therapy of allergy, where they would saturate cell-bound IgE, and prevent allergen interaction and therefore cross-linking and activation of mast cells (Valenta et al., 1998).

Phage display libraries have been used to isolate mimotopes of several allergens, including the major house dust mite allergen Der p 1, the birch allergen Bet v 1 and grass allergens.

1.5.2.1 Bet v 1 Mimotopes

Extensive work has been completed in the investigation of mimotopes of the major birch allergen Bet v 1, resulting in the most comprehensive assessment of mimotopes to date. Jensen-Jarolim et al. (1998) generated two Bet v 1 mAbs, BIP1 and BIP4 by standard protocols, and used these two antibodies to screen phage display libraries for mimotopes of Bet v 1. Two filamentous phage display libraries were screened, one
expressing constrained random nonamers, and the other unconstrained random nonamers. Individual phage clones were assessed via colony lifts and an inhibition ELISA before being sequenced.

Clones isolated with one of the mAbs did not demonstrate cross-reactivity to the other mAb. BIP1 was the most successful, resulting in the isolation of 7 of 10 clones which shared an identical sequence. BIP4 panning only resulted in the isolation of a single clone. Both peptide sequences were compared to the linear and three-dimensional structure of Bet v 1; the BIP1 sequence displayed homology to a loop region, but no homology was seen for the BIP4 sequence. The conformation of synthetic peptides representing the two isolated sequences was investigated using nuclear magnetic resonance (NMR) spectroscopy; both were conformationally unstable in solution. The synthetic peptides were incapable of inhibiting mAb-Bet v 1 reactivity, probably due to the conformational instability. In contrast, the phage-displayed peptides were capable of inhibiting the reaction.

The immunogenicity of the BIP1 reactive peptides was investigated by administering phage-displayed peptide via the gastric route without adjuvant. Sera was assessed via immunoblotting and two of the group of four mice found to be producing antibodies capable of cross-reacting to whole Bet v 1. This confirmed that the BIP1 reactive peptides were indeed mimotopes, and that phage displayed peptides were capable of raising an IgG response. The potential of this IgG to block allergen-IgE interactions was not investigated.

Further work with these Bet v 1 mimotopes was carried out by Jensen-Jarolim (1999). The BIP1 mimotope, termed Bet mim 1, was administered to mice via the oral route before the mice were systemically sensitised with Bet v 1 in alum. Administration of the Bet mim 1 phage was shown to increase allergic skin reactivity to Bet v 1, an effect that correlated with the action of the BIP1 mAb, which also enhances the binding of IgE to Bet v 1. The enhancement of skin reactivity was dependent on the mimotope, and not the phage carrier.

Mimotopes of Bet v 1 have also been isolated by biopanning phage display libraries with purified anti-Bet v 1 IgE from allergic patients. IgE was purified from a pool of 12 allergic patient’s serum and used as the target for biopanning of two phage display
libraries, one displaying random constrained nonamers, and the other displaying unconstrained random nonamers. Sequencing of the isolated phage clones revealed a consensus sequence which was isolated to an exposed region on the three-dimensional model of Bet v 1, though no similarity to the linear sequence was seen. The dominant clone, termed Bet mim E, was used to immunise mice via the intraperitoneal route without the use of adjuvant. Immune serum was used in an inhibition assay, and was shown to be capable of inhibiting the interaction of mouse Bet v 1 immune serum with Bet v 1. Bet mim E immune serum was also capable of inhibiting the binding of human IgE to Bet v 1, whereas Bet mim 1 (Jensen-Jarolim et al., 1998) was not. The epitopes which are mimicked by these two mimotopes were located at a substantial distance from each other on the Bet v 1 molecule. While the Bet mim 1 mimotope acted to enhance immune reactivity, the Bet mim E mimotope raised IgG antibodies that were capable of blocking the interaction of IgE and Bet v 1 – blocking antibodies.

One of the concerns of the use of phage display is that phage vectors are unsuitable for use in a therapeutic setting. Being multivalent vectors, they would obviously be capable of cross-linking cell-bound IgE, resulting in anaphylaxis. It is therefore envisaged that mimotopes would require being expressed in another system in order to be of use in humans. Ganglberger et al. (2001) produced one of the Bet v 1 mimotopes isolated from IgE biopanning, Bet mim E, as a monovalent fusion with streptococcal albumin binding protein (ABP). This fusion protein was used to immunise mice via the intraperitoneal route with alum as an adjuvant. Immune serum was assessed via ELISA, inhibition assay and reactivity in a skin hypersensitivity assay.

The fusion proteins were able to bind anti-Bet v 1 IgE, indicating that the conformation of the epitope was conserved. Mice immunised with the fusions produced IgG antibodies capable of recognising Bet v 1, while the fusion protein itself was able to inhibit the binding of IgE to Bet v 1. Neither of the fusion proteins induced skin hypersensitivity or any other detrimental side effects, even one year after being administered. It was concluded that these fusion proteins were safe for use in immunotherapy, being able to induce blocking antibodies but not anaphylaxis.

The Bet mim 1 mimotope was further investigated and shown to mainly induce cross-reactive IgG1 capable of recognising Bet v 1 when administered to mice as phage-displayed peptides via the intraperitoneal route, without adjuvant. All other isotypes –
IgE, IgG2a, IgG2b and IgA – were undetectable. Spleen cells from immunised mice could not be stimulated with Bet v 1 or a free peptide representing the Bet m 1 sequence. Bet v 1 specific T cells from allergic patients could not be stimulated with the phage-displayed or synthetic mimotope. The application of the mimotopes to the induction of tolerance was investigated; neither phage-displayed or synthetic mimotopes were capable of inducing tolerance in mice when delivered via the intranasal route (Scholl et al., 2002). These results indicate that the mimotopes themselves do not cross-react with the natural allergen at the T cell level, but rather that compounds within the phage itself activate T cells that help B cells to generate an antibody response against the mimotope. Further, the mimotope identified represents solely a B cell epitope.

1.5.2.2 Der p 1 Mimotopes

Mimotopes have been isolated for the major house dust mite allergen Der p 1 by Furmonaviciene et al. (1999). This study employed a mouse monoclonal antibody, 2C7, which had previously been recognised as binding to the same epitope on Der p 1 as human IgE (McElveen et al., 1998). 2C7 binding to Der p 1 was also inhibited by the mouse mAb 4C1, which is cross-reactive between Der p 1 and Der f 1, and capable of partially blocking IgE binding to Der p 1 (Chapman et al., 1987). The genes encoding 2C7 were sequenced by McElveen et al. (1998) and found to demonstrate greater than 70% homology with human immunoglobulin V-gene segments. The interaction of 2C7 with Der p 1 was also modelled by comparison with existing immunoglobulin structures. Due to the existence of this model, as well as the fact that 2C7 was capable of inhibiting human IgE binding to Der p 1, it was selected as a target for mimotope isolating from phage display libraries.

Two different phage display libraries were employed by Furmonaviciene et al. (1999) to isolate mimotopes of Der p 1. The libraries used were a random pentadecamer pVIII filamentous phage display library (f884) and a constrained random nonamer T7 phage display library. Biopanning with 2C7 isolated phage clones from both libraries which shared a consensus sequence. Phage clones were assessed via ELISA or plaque lift. This consensus demonstrated similarity to the linear sequence Leu147-Gln160 in the
native Der p 1 sequence. This sequence was located on the three-dimensional model of Der p 1 on a loop (Val140-Tyr169) in a region of $\beta$-sheets.

Further investigation into the relevance of this epitope was carried out by assessing the ability of mAb 2C7 to recognise the homologous cysteine proteases chymopapain, papain and actinidin. Papain and chymopapain were recognised by 2C7, but actinidin was not. The authors concluded that the extent to which 2C7 bound to the cysteine proteases was directly related to the solvent accessibility of the epitope corresponding to Leu147-Gln160 of Der p 1.

One of the major drawbacks to this study is that the immunogenicity of the isolated mimotopes was not investigated. As stated previously, the ability of raising an antibody response that recognises the full antigen is a critical property. The alignment with the native Der p 1 sequence appeared to result in a direct alignment with the mimotopes; again, it is not always expected that a mimotope would share the same residues as the native sequence, due to the peptide essentially conforming to the same shape as the native epitope. A direct alignment with the native protein does not indicate, therefore, that an important epitope has been defined. The authors concluded that they had identified a cross-reactive epitope representative of the human and mouse anti-Der p 1 response. Immunological data is required in order for this conclusion to be verified.

Furthermore, the conclusion that the mAb 2C7 represents a major component of the human IgE response against Der p 1 is based on a small experiment only. McElveen et al. (1998) assessed the ability of 2C7 to inhibit binding of human allergic serum by a direct comparison with the mAb 4C1. Only a small group of allergic sera was assessed, with no information on variables such as their RAST values given. A larger study is required to confirm the conclusion that the specificity recognised by this mAb indeed represents a component of the human IgE response.

1.5.2.3 Grass allergen mimotopes

Grass pollen allergy affects 25-30% of the population, with many allergens from different species of grass displaying cross-reactivity. This cross-reactivity was used to advantage for the isolation of mimotopes of grass pollen allergens (Suphioglu et al.,
A mixture of grass extracts, including rye (*Lolium perenne*), Bermuda (*Cynodon dactylon*) and timothy (*Phelum pratense*) grasses, was used to generate mAbs in BALB/c mice. A mAb, 2A1, which demonstrated binding to a number of grass pollen allergens, was then used to screen a pIII displayed random dodecamer library (PhD12). The isolated phage were sequenced, and the mimotope sequence then produced as a synthetic peptide. The mimotope displayed homology to a linear portion of Lol p 1, the major allergen from rye grass, which was part of a sequence conserved between group one grass allergens.

The synthetic peptide form of the mimotope was able to inhibit the binding of the mAb 2A1 as well as allergic human serum binding to grass pollen extract. The cross-reactivity of the mAb was also investigated; the antibody bound to the group one allergens from several different grass species, indicating that it recognised a cross-reactive epitope. The mAb also was able to inhibit the binding of allergic serum to rye grass pollen extract, indicating that it shared an epitope with human IgE.

This study concluded that they had identified a cross-reactive mimotope which represented an epitope recognised by murine mAbs and human IgE, and could potentially be exploited for the generation of blocking antibodies. Unfortunately, there were no immunisation studies carried out with either the phage-displayed or a synthetic mimotope, so the immunogenicity of this sequence is unknown.

Mimotopes of the timothy grass pollen allergen have also been employed in order to map conformational IgE epitopes of Phl p 5a (Hantusch et al., 2004). Anti-Phl p 5a IgE was used to biopan a constrained decapeptide phage display library, and the isolated phage clones analysed for affinity and immunogenicity. The phage-displayed peptides displayed similarity to three different regions of the Phl p 5a primary sequence, all of which were identified on the three-dimensional structure of the allergen. The phage clones were also able to inhibit the binding of IgE to Phl p 5a, as well as induce the production of anti-Phl p 5a antibodies in mice. The authors concluded that the mimotopes represented three different IgE epitopes on Phl p 5a.
1.5.2.4 Profilin mimotopes

Mimotopes have also been isolated for another important birch allergen, the profilin Bet v 2 (Leitner et al., 1998). Around 20% of pollen and food allergic individuals produce IgE recognising profilins. These antibodies are highly cross-reactive with profilins from different plants; the proteins themselves are highly conserved.

IgG and IgE directed against profilins from birch pollen, mugwort pollen and celery tuber was isolated from allergic serum. This purified serum was used in a series of biopanning experiments against two pVIII random nonamer display libraries, one of which expressed disulphide-constrained peptides. Rounds of panning were performed against anti-mugwort profilin purified serum, then anti-birch profilin serum, anti-celery tuber profilin serum and finally anti-mugwort profilin purified serum again. This strategy was employed specifically to enrich for phage displaying peptides representative of cross-reactive epitopes between the species.

The isolated phage were sequenced and found to express a sequence that did not display any homology with the linear or three-dimensional model of birch profilin, including known IgE binding regions. A synthetic peptide representing the most commonly isolated sequence was capable of inhibiting the binding of IgE to profilins from mugwort, birch and celery profilins. Together, these results indicate that this mimotope represents a cross-reactive epitope on profilins, mimicking the shape of binding, and not the direct sequence.

As in the studies on Der p 1 and grass pollen mimotopes, no immunisation studies were performed, so the immunogenicity is unknown.

1.6 EXPERIMENTAL AIMS OF THE THESIS

Phage display libraries have previously been used to isolate mimotopes of many different proteins, among them allergens of house dust mites, birch and grass. Several of these mimotopes have been immunogenic, capable of raising antibodies against the original antigen. In the case of Bet v 1 mimotopes, the peptides isolated were capable of raising blocking antibodies against Bet v 1. Many of the other studies have been
limited in that they have not followed through with immunisation experiments, so it is
difficult to determine the relevance of the mimotopes isolated. Furthermore, the
immunisation experiments that have been carried out to date have all used different
methodology, making it difficult to compare the different studies.

The aims of the experiments contained in this thesis are to use phage display libraries to
isolate mimotopes representing the model allergen papain, the model epitope FLAG and
the house dust mite allergens Der p 1 and Der p 2. Papain was chosen as a model
because a test system of respiratory sensitisation has been previously developed in the
laboratory (Lenzo et al., 2004), facilitating in vivo analysis of the mimotopes. Anti-
papain antibodies will be first analysed for their ability to block sensitisation to papain
and then used to screen phage display libraries for papain mimotopes.

Variants of the FLAG epitope will be isolated from phage display libraries, and these
peptides used in pilot experiments. The immunogenicity of these variants will be
investigated, as well as the effect of factors such as route of delivery and adjuvant
present on the antibody response to mimotopes. The isolation of Der p 2 mimotopes
will follow. These mimotopes will be examined both in vitro and in vivo, and will be
cloned as monovalent fusions and examined for immunogenicity.
CHAPTER TWO

MATERIALS
2.1 BUFFERS AND SOLUTIONS

Annealing Buffer
Tris pH 7.5-8.0 10mM
NaCl 50mM
EDTA 1mM

Blocking Buffer
Tris-HCl pH 7.4 1M
BSA 0.5% (w/v)
Stored in 50ml aliquots at -20°C.

Carbonate Coating Buffer
Na$_2$CO$_3$ 100mM
NAHCO$_3$ 100mM
pH 9.6. Stored in 50ml aliquots at -20°C.

IPTG
IPTG 200µg/ml
In sterile water, filter sterilised and stored at -20°C.

2M Mg++
MgCl$_2$ 1M
MgSO$_4$ 1M
Filter sterilised.

PEG/NaCl (PhD Library)
PEG 8000 20% (w/v)
NaCl 2.5M
Filter sterilised or autoclaved for larger volumes.

PEG/NaCl (f884 library)
PEG 8000 16.7% (w/v)
NaCl 3.3M
Filter sterilised or autoclaved for larger volumes.
**Phosphate Buffered Saline**

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<td>Na$_2$HPO$_4$</td>
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<td>KH$_2$PO$_4$</td>
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Adjusted to pH 7.4 with HCl

**Phage Storage Buffer**

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**Potassium Phosphate Buffer**

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Autoclaved

**TBS**

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**TRF Wash Buffer**

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<tr>
<td>Tween 20</td>
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</tbody>
</table>
X-gal 20mg/ml
In dimethylformamide. Stored at -20°C.

### 2.2 MEDIA

All media was sterilized by autoclaving except where noted.

#### 2.2.1 Liquid Media

**Luria Broth (LB)**

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**SOC**

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<tr>
<td>NaCl</td>
<td>0.58g/L</td>
</tr>
<tr>
<td>KCl</td>
<td>0.19g/L</td>
</tr>
</tbody>
</table>

Autoclaved in 100mL aliquots then 1mL of 2M Mg++ and 1mL 2M glucose (filter sterilised) added.

**Terrific Broth**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>12g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>24g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>4ml (5.04g)</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>900ml</td>
</tr>
</tbody>
</table>

Autoclaved in 90ml aliquots. When cooled, 10mL of potassium phosphate buffer was added.

**2x YT**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>16g/L</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10g/L</td>
</tr>
<tr>
<td>NaCl</td>
<td>5g/L</td>
</tr>
</tbody>
</table>
2.2.2 Solid Media

LB Agar
LB agar was prepared by 1.5% agar to LB broth.

Top Agarose
Tryptone 1% (w/v)
NaCl 0.8% (w/v)
Agarose 0.6% (w/v)

2.2.3 Cell Culture Media

DMEM
D-MEM (Dulbecco’s Modified Eagle media) was purchased as a powder (Life Technologies Ltd., Madison, U.S.A.) and reconstituted as per the manufacturer’s instructions and filter sterilized. Gentamicin and glutamine were added. Foetal calf serum (FCS; Trace Scientific, Melbourne, Australia) was added to 20% (v/v) when required.

2.3 VECTORS AND PHAGE LIBRARIES

f884 Phage Display Library
Random peptide library on the filamentous bacteriophage fd expressing random 15 amino acid peptides at ~150 copies per virion on protein VIII.
Source: Professor George Smith, Missouri, USA.

PhD Phage Display Libraries
Random peptide libraries on the filamentous bacteriophage M13KE. PhD7 expresses random heptamers, PhD12 random dodecamers, PhDC7C disulphide constrained heptamers. The recombinant peptide was expressed on protein III at five copies per virion.
Source: New England Biolabs (Beverly, USA).

**Phylomer Library**
Fragments of bacterial genomes were cloned into the T7-415 vector, which expressed 415 copies of the recombinant peptide per virion.
Vector source: Novagen (Madison, USA).

### 2.4 BACTERIAL STRAINS

**E. coli BL21**
Genotype: F- ompT hsdS\(B^{rB-}\) m\(B^{-}\) gal dcm

**E. coli K12 ER2738**
Genotype: F\(^{'}\) pro\(A^{+}\) B+ lacI q\(\Delta(lacZ)\) M\(15\) zzf::Tn 10(Tet R)/ fhuA2 glnV \(\Delta(lac-proAB)\) thi-1 \(\Delta(hsdS-mcrB)\) 5

**E. coli K91**
Genotype: thi

**E. coli MC1061**
Genotype: hsdR mcrB araD\(139\) D(araABC-leu)\(7679\) D(lac)\(174\) galU galK strA thi
2.5 Oligonucleotides

The oligonucleotides used for PCR and sequencing are shown in table 2.1. All oligonucleotides were synthesised by Geneworks, Adelaide, Australia.

<table>
<thead>
<tr>
<th>Oligonucleotide Name and Use</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>-96 (PhD sequencing)</td>
<td>CCCTCATAGTTAGCGTAACG</td>
</tr>
<tr>
<td>f884 forward (f884 sequencing)</td>
<td>CTTTCCCCGTCAAGCTCTAAATC</td>
</tr>
<tr>
<td>f884 reverse (f884 sequencing)</td>
<td>AACGATAAACAACCACCATAGCCC</td>
</tr>
<tr>
<td>M13ext (PhD sequencing)</td>
<td>CATGCCGGGTACCTTTCTATTTCTC</td>
</tr>
<tr>
<td>T7 forward (T7 sequencing)</td>
<td>CGTATTCCAGTCAGGTGTGATGC</td>
</tr>
<tr>
<td>T7 reverse (T7 sequencing)</td>
<td>ACCCCTCAAGACCCTGTAGAG</td>
</tr>
</tbody>
</table>

Table 2.1 Oligonucleotide primers.

2.6 Seras

Serum was collected from volunteers and stored at -20°C for no more than six months before use. The skin prick test reactivity and RAST scores of these individuals were previously determined.
2.7 ANTIBODIES

The antibodies used in biopanning and time-resolved fluorescence (TRF) assays are shown in table 2.2.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>10A3</td>
<td>Papain</td>
<td>In house</td>
</tr>
<tr>
<td>3D5</td>
<td>Papain</td>
<td>In house</td>
</tr>
<tr>
<td>8E11</td>
<td>Papain</td>
<td>In house</td>
</tr>
<tr>
<td>9B5</td>
<td>Papain</td>
<td>In house</td>
</tr>
<tr>
<td>4C1</td>
<td>Der p 1</td>
<td>Indoor Biotechnologies (Cardiff, UK)</td>
</tr>
<tr>
<td>10B2</td>
<td>Der p 2</td>
<td>In house</td>
</tr>
<tr>
<td>T7-Tag (Biotin, HRP)</td>
<td>T7 bacteriophage</td>
<td>Novagen</td>
</tr>
<tr>
<td>FLAG M2 (Biotin)</td>
<td>FLAG peptide</td>
<td>Sigma (St Louis, USA)</td>
</tr>
<tr>
<td>Anti-bacteriophage fd(Biotin)</td>
<td>Filamentous bacteriophage</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-bacteriophage M13, HRP conjugate</td>
<td>Filamentous bacteriophage</td>
<td>Amersham Pharmacia (Uppsala, Sweden)</td>
</tr>
<tr>
<td>Goat anti-mouse IgG1</td>
<td>Mouse IgG1</td>
<td>Southern Biotechnology Associates (Birmingham, USA)</td>
</tr>
<tr>
<td>Goat anti-mouse IgG2a</td>
<td>Mouse IgG2a</td>
<td>Southern Biotechnology Associates</td>
</tr>
<tr>
<td>Rabbit anti-mouse IgG, biotinylated</td>
<td>Mouse IgG</td>
<td>Amersham Pharmacia</td>
</tr>
</tbody>
</table>

Table 2.2 Antibodies used in biopanning and TRF assays. Brackets denote variants of the antibody also used; these have the same target and supplier.
2.8 PROTEINS AND PEPTIDES

Der p 1
Natural Der p 1 (nDer p 1) was isolated from spent mite medium (SMM) by affinity chromatography using anti-Der p 1 mAb conjugated to Sepharose.

Der p 2
Natural Der p 2 (nDer p 2) was isolated from spent mite medium (SMM) by affinity chromatography using anti-Der p 2 mAb conjugated to Sepharose. Recombinant Der p 2 (rDer p 2) was produced as a GST fusion in *E. coli* in the pGEX vector, or in *Pichia pastoris*.

Papain
Papain was purchased from Sigma and resuspended in ddH$_2$O before use.
CHAPTER THREE

METHODS
3.1 PhD PHAGE DISPLAY LIBRARY

3.1.1 Biopanning

A 35mm polystyrene tissue culture dish (Nunc, Denmark) was coated with 10μg of the target monoclonal antibody in 100μL of 0.1M NaHCO$_3$ pH 8.6 overnight at 4°C. The plate was blocked with 3% bovine serum albumin (BSA; JRH Biosciences, Lenexa, USA) for two hours at room temperature (RT). An appropriate titre of bacteriophage diluted in 3% BSA was added and allowed to react overnight at 4°C. The plates were washed four to ten times with TBS + 0.05% Tween before bound phage were eluted with 400μl of 0.2M glycine-HCl pH 2.2. The eluate was neutralised with 75μl of Tris-HCl pH 9.1, titred (section 1.1.2) and stored at 4°C.

An aliquot of the original PhD7, PhD12 or PhDC7C libraries (New England Biolabs, Beverly, USA) or a combination of the three libraries was used as the input for the first round of biopanning. The phage-containing eluate from biopanning was amplified as per section 3.1.3, and 100μl of this amplified eluate was then used as the input for the subsequent round of biopanning. Three to four rounds of biopanning were performed, or until a significant increase in the recovery of phage was observed.

3.1.2 Titration

A fresh overnight culture of *Escherichia coli* ER2738 (New England Biolabs) was diluted in Luria broth (LB) and incubated at 37°C and 225rpm until the bacteria had reached mid-log phase (OD$_{600nm}$~0.5). Phage was serially diluted in LB, 10μl of each dilution added to 200μl of mid-log ER2738 and incubated at 37°C for 15 mins. The infected culture was added to 3mL of molten top agarose to which 40μl of X-gal and 16μl of IPTG had been added, poured onto pre-warmed LB agar plates, allowed to set and incubated overnight at 37°C. Plaques were counted on plates containing 30-300 plaques and the titre calculated.
3.1.3 Small Scale Amplification of Phage

A fresh overnight culture of *E. coli* ER2738 was diluted 1:100 in LB. For amplification of the eluate from the first round of biopanning, the total eluate was added to the diluted culture, whereas for later rounds, 100μl of the eluate was added. The culture was incubated at 37°C and 225rpm for 4.5 hours before the bacteria were pelleted for 10 mins at 10,000rpm and 4°C (Sorvall SS-34 rotor). The supernatant was transferred to a fresh tube and clarified again by centrifugation.

The upper 80% of the supernatant was transferred to a fresh tube and 1/6th volume of PEG/NaCl added, mixed well and incubated at 4°C for at least 60 mins. The phage were pelleted at 10,000rpm for 15 mins at 4°C (Sorvall SS-34 rotor) and the supernatant removed. The pellet was resuspended in phage storage buffer (PSB), clarified by centrifugation and stored at 4°C.

3.1.4 Large Scale Amplification of Phage

A fresh overnight culture of *E. coli* ER2738 was diluted 1:100 in 2 x YT to a volume of 500ml. Twenty microlitres of high titre phage stock was added to the culture, which was then incubated overnight at 37°C and 225rpm. The bacteria were spun down at 7,500rpm at 4°C (Sorvall GS-3 rotor) for 30 mins, the supernatant precipitated by the addition of 1/6th volume of PEG/NaCl and incubation overnight at 4°C.

The precipitation reaction was centrifuged at 7,500rpm at 4°C (GS-3 rotor) for 30 mins and the pellet resuspended in 40ml PBS. The phage were re-precipitated with 1/6th volume of PEG/NaCl at 4°C for 60 mins, centrifuged for 10 mins at 10,000rpm (Sorvall SS-34 rotor) and resuspended in an appropriate buffer before being stored at 4°C.
3.1.5 Phage Plaque Preparation for Screening

Phage clones were plated via agar overlay (section 3.1.2) at a dilution resulting in the growth of well-isolated plaques (10-50 plaques per plate). A plug of agar containing a plaque separated by at least 1cm from other plaques was picked with a sterile plastic Pasteur pipette and deposited in 200μl of PSB and stored at 4°C.

Ten microlitres of the phage plug stock was added to 2ml of a 1:100 dilution of overnight *E. coli* ER2738 in 2x YT and amplified at 37°C 225rpm for 4.5 hours. The bacteria were pelleted in a microfuge at 13,000rpm for 10 mins, and the clarified supernatant stored at 4°C. The phage-containing supernatant was used immediately in a time resolved fluorescence (TRF) assay.

3.1.6 Phage Time-Resolved Fluorescence Assay

The dissociation enhanced lanthanide fluoroimmunoassay (DELFIA) was used to detect phage binding to antibodies. Ninety-six well Maxisorp plates (Nunc) were coated overnight at 4°C with 100μl/well of 1μg/ml target antibody or isotype control antibody in carbonate coating buffer. The coating solution was discarded and the plates blocked by addition of 200μl/well of blocking buffer at RT for 1-2 hours. One hundred microlitres of the phage containing supernatant was added per well, and allowed to react overnight at 4°C. The plates were washed with TRF wash buffer and specifically binding phage detected by incubation with biotinylated anti-fd bacteriophage mAb (Sigma, St Louis, USA) diluted in assay buffer (Wallac, Turku, Finland), and then streptavidin-europium (Wallac). Finally, 150μl of enhancement solution (Wallac) was added per well, the plates incubated for at least 15 mins and then read using a Wallac Victor 2 counter (Wallac) at 615nm.
3.1.7 Phage Enzyme-Linked Immunosorbent Assay

Phage clones were amplified as per section 3.1.5. Maxisorp plates were coated overnight at 4°C with 100μl/well of 1μg/ml target antibody or isotype control antibody in carbonate coating buffer. The wells were blocked with 200μl of 3% BSA in phosphate buffered saline (PBS). One hundred microlitres of phage-containing supernatant was reacted per well overnight at 4°C. The plates were washed with PBS + 0.05% Tween and specifically binding phage detected with horseradish peroxidase (HRP) labelled anti-M13 monoclonal antibody (Amersham Pharmacia, Uppsala, Sweden). The plates were washed and 100μl of the substrate TMB (Graphic Scientific, 10 Brisbane, Australia) was added. Plates were read at 630nm using a Microplate EL 311 autoreader (Bio-Tek Instruments) or 50μl of 1M H2SO4 was added per well to stop the reaction and the plates then read at 450nm.

3.1.8 Phage Competition Assay

Competition assays were performed via a TRF (section 3.1.5) or ELISA (section 3.1.6) assay. These assays were performed as per the screening methods, except that a competing protein was added in varying concentrations at the same time as the phage supernatant was added.

3.1.9 Phage ssDNA Isolation

Individual phage clones were purified by plating for single plaques (section 3.1.2). A well isolated plaque was picked using a sterile disposable Pasteur pipette and inoculated into 2ml of overnight E. coli ER2738 diluted 1:100 in 2 x YT. This culture was incubated at 37°C and 225rpm for 6 hours. Bacteria were pelleted by centrifugation in a microfuge at 13,000rpm for 10 mins. One millilitre of phage-containing supernatant was added to 270μL of PEG/NaCl, mixed and incubated at RT for 15 mins before the phage were pelleted in a microfuge at 13,000rpm for 5 mins. The dried pellet was resuspended in 200μL of TE buffer.
The phage were then treated by phenol extraction to remove the protein component. Briefly, 200μL of TE saturated phenol was added, the tube vortexed for 1 min and then spun in a microfuge at 13,000rpm for 2 mins. The upper aqueous phase was removed to a fresh tube, and the protein-containing organic phase discarded. The aqueous phase was re-extracted with Leder phenol (phenol : chloroform : isoamyl alcohol at 25:24:1) and then chloroform before being ethanol precipitated.

DNA was precipitated by adding 1μl of glycogen, 2.5 volumes of 100% ethanol and 0.1 volumes of 3M sodium acetate, incubating on ice for 15 min and then centrifuging for 10 min at 13,000rpm. The DNA pellet was washed with 70% ethanol and air dried before being resuspended in TE buffer.

The isolated DNA was sequenced via automated cycle sequencing on an ABI Prism 3730 48 capillary sequencer (Dept. of Clinical Immunology, Royal Perth Hospital, Perth, Western Australia) using dye-terminator reactions. Nucleotide and protein sequence analysis were performed using MacVector software (Oxford Molecular Group, Oxford, UK).

### 3.1.10 Cesium Chloride Gradient

Phage were amplified as per section 3.1.4 and resuspended in 8mL of TE buffer. Cesium chloride was added to a final concentration of 0.5mg/mL and density gradient centrifugation carried out. The gradients were spun at 35,000rpm in the Sw41Ti rotor (Beckman) for 22 hours at 15°C. The phage band was collected with a syringe and dialysed against TE buffer overnight at 4°C.
3.2 f884 PHAGE DISPLAY LIBRARY

3.2.1 Preparation of Electrocompetent Bacteria

The f884 phage display library was obtained as a replicative form (RF) DNA stock. For use, it was necessary to transform the library DNA into electrocompetent *E. coli* MC1061 cells.

A fresh overnight culture of *E. coli* MC1061 was used to inoculate 500mL of LB and grown at 37°C 225rpm until the culture had reached an OD$_{600nm}$ of ~0.5. The culture were chilled in ice water, and kept as cold as possible during the later steps. The bacteria were pelleted by centrifugation at 2000rpm (Sorvall GSA rotor) for 15 mins at 4°C, and the supernatant decanted. The pellet was gently resuspended in 150mL ice cold 1mM HEPES, and re-pelleted. This procedure was repeated using 60ml 1mM HEPES, then 10mL of ice cold 10% glycerol. The bacteria were pelleted at 3000rpm for 10 mins at 4°C (Sorvall SS-34 rotor), the supernatant decanted and the pellet resuspended in 500μL of ice-cold 10% glycerol. The electrocompetent cells were aliquotted into 1.5mL microfuge tubes, snap frozen by immersion in liquid nitrogen, and stored at -80°C.

3.2.2 Electroporation

The f884 RF DNA was electroporated into several tubes of electrocompetent *E. coli* MC1061, adding 8μg of DNA per 200μL cells. The electrocompetent cells were thawed, and DNA added and mixed with a pipette tip before incubation on ice for 1-2 mins. The mixture was pipetted into an ice-cold 0.2cm electroporation cuvette which was placed on ice briefly before being pulsed in a BioRad Gene Pulser electroporator (2.5kV, 25μF, 400Ω). Two millilitres of SOC medium was added to the cuvette to suspend the cells and then transferred to a 15mL tube. The culture was incubated at
37°C 225rpm for 1 hour and then titred. A working stock of phage was maintained at 4°C and aliquots of the library were stored at –80°C.

3.2.3 Biopanning

Biopanning of the f884 library was performed in the same manner as the PhD libraries (section 3.1.1).

3.2.4 Titration

Titration of f884 phage cultures was performed using *E. coli* K91 as a host strain. A fresh overnight culture of *E. coli* K91 was used to inoculate 10mL of terrific broth, which was grown at 37°C and 225rpm until the OD$_{600nm}$ of a 1/10 dilution reached ~0.2. The culture was used immediately.

Dilutions of phage were made in TBS + 1% gelatine. Ten microlitres of the K91 terrific culture was added to the 10μL of phage dilution, mixed, and incubated at RT for 10 mins. One millilitre of LB + 0.2μg/ml tetracycline was added and the cultures incubated at 37°C for 20-40 mins. Two hundred microlitres of this mixture was spread onto dried LB agar plates containing 40μg/mL tetracycline, the plates incubated at 37°C overnight and colonies counted.

3.2.5 Small Scale Amplification

For the first round of biopanning, the entire eluate was concentrated and amplified. Concentration was achieved by spinning in a Centricon 30kDa ultrafilter (Millipore, Billerica, USA), washing once with TBS, and then re-centrifuging to give a final volume of ~100μL. This eluate, or 100μL of later round eluates, was mixed with 100μL of *E. coli* K91 terrific culture (section 1.2.4) and incubated for 10-30 mins at RT.
The infected cells were added to 20mL of LB + 0.2μg/mL tetracycline and incubated for 30-60 mins at 37°C. Tetracycline was added to a final concentration of 20μg/mL and the culture incubated overnight at 37°C 225rpm.

Bacteria were pelleted by centrifugation for 10 mins at 8,000rpm and then 5,000rpm (Sorvall SS-34 rotor). Three millilitres of PEG/NaCl was added to the clarified supernatant, mixed and incubated overnight at 4°C. The precipitated phage were pelleted at 10,000rpm (Sorvall SS-34 rotor) and the pellet resuspended in 1mL TBS. The phage were re-precipitated with 150μL of PEG/NaCl, pelleted for 5 mins in a microfuge, and then resuspended in PBS and titered.

3.2.6 Large Scale Amplification

A single colony of *E. coli* K91 infected with the phage clone of interest was used to inoculate 2mL of LB + 20μg/mL tetracycline. This culture was incubated overnight at 37°C 225rpm. One hundred microlitres of this culture was then used to inoculate 500mL of 2xYT + 20μg/mL tetracycline, which was incubated overnight at 37°C with orbital shaking at 225rpm.

Bacteria were pelleted at 7,500rpm (Sorvall GS-3 rotor) at 4°C for 30 mins. The supernatant was precipitated by the addition of 0.15 volumes of PEG/NaCl, mixed well and then incubated overnight at 4°C. Precipitated phage were pelleted at 7,500rpm (Sorvall GS-3 rotor) as above, and resuspended in 40mL of PBS. This solution was clarified and re-precipitated with 0.15 volumes of PEG/NaCl. Pelleted phage were resuspended in an appropriate volume of TE and stored at 4°C.
3.2.7 Phage Time Resolved Fluorescence Assay and Competition Assay

Screening of individual phage clones was performed as per section 3.1.6, using the biotinylated anti-fd mAb (Sigma). Competition assays were performed as per section 3.1.8.

3.2.8 Isolation of Phage RF DNA

A single colony of *E. coli* K91 infected with the phage clone of interest was used to inoculate 5mL of LB + 20μg/mL tetracycline; the culture was incubated overnight at 37°C 225rpm. RF DNA was isolated from 1mL of this culture using a plasmid miniprep kit (Qiagen), following the manufacturer’s instructions.

3.2.9 Cesium Chloride Gradient

Cesium chloride gradients were performed using the same conditions as for the PhD library clones (section 3.1.9)

3.3 T7 PHAGE METHODS

3.3.1 Biopanning

Biopanning of the T7 phage display libraries was performed using 35mm tissue culture dishes (Nunc). The dish was coated with target antibody (10μg for the first round, 1μg
for later rounds) diluted in 500μL TBS. The dish was then blocked with 5% skim milk powder and washed with TBS before use.

An appropriate titre of T7 phage was added to the dish, diluted in TBS + 0.05% Tween to a final volume of 500μL and allowed to react overnight at 4°C. The dish was washed for five mins with TBS + 0.05% Tween, six times for the first round, and ten times for later rounds. Binding phage were eluted by the addition of 1% SDS and the eluates titred and stored at 4°C. Phage were amplified and used as the input into the next round of biopanning, for a total of three to four rounds.

3.3.2 Titration of T7 Phage

An overnight culture of *E. coli* BL21 was diluted 1:100 in LB and incubated at 37°C 225rpm until mid-log phase (OD$_{600nm}$ ~ 1.0). Serial dilutions of phage were made in LB, and 100μL of the diluted phage added too 250μL of *E. coli*. Three millilitres of molten top agarose was immediately added to the infected cells, and the resultant mixture poured onto warmed, pre-dried LB agar plates. After setting, the plates were inverted and incubated at 37°C for 3-4 hours or overnight at RT. Plaques were then counted, and the titre calculated from plates with 30-300 plaques.

3.3.3 Amplification

An overnight culture of *E. coli* BL21 was diluted 1:100 in LB and incubated at 37°C until it had reached an OD$_{600nm}$ of 0.5-1.0. One hundred microlitres of high titre phage stock was added, and the culture incubated until lysis had occurred. At this point, the culture was clarified by centrifugation for 10 mins at 10,000rpm (Sorvall SS-34 rotor) and stored at 4°C for further biopanning, or further purified.

For cultures that were further purified, NaCl was added to the culture at 100g/L before centrifugation. A solution of 50% PEG was added to the clarified supernatant at 1:6
(vol:vol), mixed by vortexing, and incubated overnight at 4°C. The precipitated phage were pelleted, resuspended in an appropriate buffer, and stored at 4°C.

### 3.3.4 T7 Phage Time Resolved Fluorescence Assay and Competition Assay

Screening of T7 phage clones was performed by a TRF assay. T7 clones were amplified on a small scale by inoculating 2mL of 1:100 overnight *E. coli* BL21 in LB with the phage clone of interest and growing to lysis. The culture was clarified, and the phage-containing supernatant used in the assay.

The assay was performed in a similar manner to section 3.1.5, except that a biotinylated anti-T7 monoclonal antibody (T7-Tag mAb, Novagen) was used to detect binding phage. Competition assays were performed using the same conditions, expect that a varying amount of a competitor was added to the plates at the same time as the phage supernatant.

### 3.3.5 T7 SDS Treatment

Phage clones were amplified as per section 3.3.3 and PEG precipitated. The pelleted phage were resuspended in a solution of TE + 1% SDS and then incubated at 70°C for 30 mins. SDS treated phage were then stored at 4°C.

### 3.3.6 T7 Cesium Chloride Gradient

Phage clones were amplified as per section 3.3.3 and PEG precipitated. The pelleted phage were resuspended in 1mL TE and layered onto a CsCl gradient.
The CsCl gradient was prepared by layering dilutions of 62.5% CsCl in the following order from the bottom to top layer: 1mL of 1:0 (CsCl:TE), 2mL of 2:1, 2ml of 1:1 and 2mL of 1:2. After addition of the phage, the gradients were centrifuged at RT for 60 mins at 35,000rpm (SW41Ti rotor). The phage band was collected with a syringe and dialysed overnight into TE to remove any remaining CsCl.

### 3.4 DNA METHODS

#### 3.4.1 Polymerase Chain Reaction (PCR)

Taq polymerase (Promega) or Pfu polymerase (Promega) were used to amplify DNA to detect the presence of an insert in a recombinant bacterial plasmid or for sequencing. Amplification was performed with 1.25 units of polymerase, 10pmoles of each primer, 5mM of each dNTP and up to 100ng of template, in a total volume or 25-50μL.

Screening of bacterial colonies was also performed by touching a sterile tip to the colony and swirling in a PCR tube containing the remaining reaction components. Phage clones were screened by adding 1-5μL of stock to the PCR.

PCRs were performed for 35-40 cycles as follows: (5 mins at 94°C); (30 seconds at 94°C, 1 min at 45-60°C depending on the calculated T_m of the primers, 1 min at 70°C) x 35-40; (7 mins at 72°C). A Perkin-Elmer 2400 or 9700 thermal cycler was used to perform all PCRs.
3.4.2 Agarose Gel Electrophoresis

Agarose gels were used to visualise DNA samples using a mini-sub gel apparatus (BioRad Laboratories, Hercules, USA). DNA grade agarose was dissolved at an appropriate concentration (1-2%) in salt-free TAE buffer, and ethidium bromide added to a final concentration of 1.5μg/mL. MetaPhor agarose (BioWhittaker, Maine, USA) was used when small fragments were to be visualised. DNA samples were diluted in 5x loading dye prior to loading and running of the gel. The DNA bands were visualised using a short wavelength UV transilluminator and visualised using the Gel Doc 2000 (BioRad).

3.4.3 Agarose Gel DNA Purification

Agarose gels were prepared as per section 3.2.2. The DNA was visualised using a long wavelength UV transilluminator, and the bands of interest excised. A gel extraction kit (Qiagen, Hilden, Germany or Eppendorf, Hamburg, Germany) was used according to the manufacturer’s instructions.

3.4.4 DNA Quantification

DNA was quantified either by direct spectrophotometry (assuming that an OD$_{260nm}$ of 1.0 = 50μg/mL dsDNA or 33μg/mL ssDNA) or via comparison with standards using the Gel Doc 2000 (BioRad).

3.4.5 Annealing of Oligonucleotides

Oligonucleotides were annealed to create cassettes to be cloned into the M13KE (New England Biolabs), f884 or T7-415 (Novagen) vectors. Complementary oligonucleotides were mixed together in an equal molar ratio in annealing buffer, incubated at 65°C for ten mins and allowed to cool slowly to RT. The oligonucleotide cassettes were gel purified on MetaPhor agarose (BioWhittaker) before used in cloning.
3.4.6 Restriction Digestion

All restriction enzymes were used according to the manufacturer’s (Promega) specifications. Reactions were performed using the supplied buffers for 4-6 hours at the appropriate temperature. The enzymes were inactivated by incubating at 65°C for 10 mins and purified by agarose gel purification (section 3.4.3) or by use of a Qiaspin PCR purification kit (Qiagen).

3.4.7 DNA Ligation

Ligations of vector and insert DNA were performed at a molar ratio of 3:1 (insert:vector). Generally, 100ng of vector DNA was used per reaction. Twenty units of T4 DNA ligase (Promega) was used per reaction in the supplied buffer, and the reactions incubated overnight at 11°C before being heated to 65°C to inactivate the ligase.

3.5 BACTERIAL METHODS

3.5.1 Chemically Competent Cells

Chemically competent cells were prepared by diluting a fresh overnight culture of the appropriate *E. coli* strain 1:100 with LB and incubating at 37°C 225rpm until an OD_{600nm} of 0.6 was reached. The bacteria were pelleted by centrifugation (5,000g, 5 mins at 4°C), resuspended in 0.5 volumes of ice-cold sterile 0.1M MgCl₂ and immediately re-pelleted. The pellet was resuspended in 0.25 volumes of ice-cold sterile 0.1M CaCl₂ and incubated on ice for 45 mins before centrifugation (5,000g, 5 mins at 4°C). The final pellet was resuspended in 0.05 volumes of ice-cold sterile 0.1M CaCl₂ + 15% glycerol, snap frozen in liquid nitrogen, and stored in aliquots at -80°C.
3.5.2 Transformation

Plasmid or phage RF DNA was incubated with 300μL of chemically competent *E. coli* on ice for 45-60 mins then heat shocked at 42°C for two mins. LB was added to a final volume of 1mL and the culture incubated at 37°C for 30 mins. The transformed bacteria were pelleted by centrifugation (2 900g, 5 mins) and 700μL of the supernatant removed. The pellet was resuspended in the remaining 300μL, and aliquots spread plated onto air dried agar plates containing the appropriate antibiotic.

3.6 MONOCLONAL ANTIBODIES

3.6.1 Immunisation Protocol

Groups of 5 C57BL/6J mice were immunised via the subcutaneous route with 100μg of antigen in a 1:1 mixture with complete Freund’s adjuvant (CFA) in a total volume of 200μl per mouse. The mice were boosted twice at 2 week intervals using incomplete Freund’s adjuvant (IFA). The mice were bled and their serum assayed for specific antibodies. One mouse was boosted with 200μg of antigen without adjuvant via the intraperitoneal route three days prior to the fusion.

3.6.2 Myeloma Cell Line

The NS-1 myeloma cell line was grown in log phase for two weeks prior to the fusion. The cells were grown in DMEM + 20% FCS in 75cm² flasks at 37°C in 7% CO₂. On the day before the fusion the cells were subcultured at a concentration of 2-3 x 10⁵cells/mL.
3.6.3 Preparation of Spleen Cells

An immunised and boosted mouse was euthanised by use of CO\textsubscript{2} gas, the spleen removed aseptically and placed into 20mL of DMEM media. The spleen cells were removed from the spleen sac by washing with a 23-gauge needle. The total number of viable cells was determined by staining with Trypan Blue.

3.6.4 Fusion of Spleen and Myeloma Cells

Myeloma and spleen cells were mixed at a ratio of 1:1 – 1:10 and centrifuged (1,000g, 5 mins). The supernatant was removed and the cell pellet loosened by gentle tapping. The cell mixture was then placed into a 37°C waterbath and 1mL of pre-warmed 50% PEG 6000 (Sigma) added drop-wise over one min with gentle mixing. The cells were rested at 37°C for one min before adding 1mL of warm DMEM drop wise over one min. Twenty millilitres of warm DMEM was then added over five mins.

The cells were pelleted by centrifugation (1,000g, 5 mins) before being resuspended in 50mL of DMEM + 20% FCS, placed in a 75cm\textsuperscript{3} flask and incubated overnight at 37°C in 7% CO\textsubscript{2}. Forty-eight millilitres of DMEM + 20% FCS and 2mL of 50x HAT were added to the cells before plating out onto ten 96-well flat bottom plates at 100\textmu L/well. The plates were incubated at 37°C 7% CO\textsubscript{2} until at least 50% confluent. The media was replaced 2-3 days before screening.

3.6.5 Screening of Hybridomas by ELISA

Maxisorp plates (Nunc) were coated with 1\mu g/mL target or control protein in carbonate coating buffer. The plates were blocked with 3% BSA in PBS + 0.05% Tween (PBS-T) and washed with PBS-T. Supernatant from the hybridoma plates was added to the Maxisorp plates, and fresh media added to the hybridomas. The Maxisorp plates were incubated at RT for 1-2 hours before washing with PBS-T. Rabbit anti-mouse Ig HRP conjugate diluted in blocking buffer was added to the plates and reacted for one hour at RT. The plates were washed with PBS-T and K-Blue TMB substrate added allowed to
develop. The plates were read at 630nm using a Microplate EL 311 autoreader (Bio-Tek Instruments) or the reaction stopped by the addition of 1M H$_2$SO$_4$ and then read at 450nm.

### 3.6.6 Cloning of Hybridomas

The wells with the highest absorbances in the ELISA were cloned by limiting dilution. Feeder spleen cells were harvested from C57BL/6J mice and used to seed the wells of flat-bottomed 96-well plates at $10^5$ cells/well. Hybridoma cells were added to the feeder cells at a dilution of 0.3 cells/well and 3 cells/well.

The hybridomas were grown to 50% confluency, expanded and screened again. Cloning was repeated until all of the clones screened at a high dilution showed the specific antibody. The cells were stored in FCS + 10% DMSO at -80°C.

### 3.6.7 Isotyping

Isotyping of monoclonal antibodies was performed either by TRF or by use of an ImmunoType strip assay (Sigma). For TRF, plates were coated with 1μg/mL of antigen, blocked with blocking buffer and hybridoma supernatant added and allowed to react at RT for 1 hour. Detection was performed with specific biotinylated antibodies directed against mouse IgG1, IgG2a, IgG3 and IgM (Southern Biotechnology Associates, Birmingham, USA or Caltag Laboratories, Burlingham, USA). Streptavidin-europium was allowed to react, and the plates developed by the addition of enhancement solution (Wallac). All antibodies were diluted in assay buffer (Wallac). Plates were read on a Wallac Victor-2 instrument at 615nm.

### 3.6.8 Biotinylation of Monoclonal Antibodies

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The antibody was dialysed against 0.1M NaHCO₃ for at least two hours at RT and the concentration adjusted to 1mg/mL. NHS-biotin was added at a ratio of 1:10-1:5 (NHS-biotin: antibody) and the reaction incubated overnight at 4°C. The biotinylated antibody was dialysed against PBS and then stored in aliquots at -20°C. Biotinylation was assessed in a TRF assay, coating a 96-well plate with the biotinylated antibody, reacting streptavidin-europium and detecting with enhancement solution (Wallac) before reading on a Wallac Victor-2 instrument at 615nm.

3.7 ANIMAL METHODS

3.7.1 Strains

Animals were all sourced from the Animal Resources Centre (Canning Vale, Western Australia). The standard mouse strains used were C57BL/6J, BALB/c and CBA/CaH, and animals were six to eight weeks of age. The histocompatibility loci of these mice were H-2ᵇ, H-2ᵈ and H-2ᵏ, respectively. All animals were maintained under ethical guidelines specified by the National Health and Medical Research Council (NM&MRC) and specific pathogen free conditions.

3.7.2 Immunisation Protocols

Immunisations were delivered either via the subcutaneous or intraperitoneal routes. For subcutaneous injections, the antigen was mixed with a 1:1 ratio of CFA or IFA to a volume of 200μl, whereas for intraperitoneal injections the antigen was delivered in an appropriate buffer, with no adjuvant, or in combination with alum (Amphogel, Whitehall Laboratories, NSW, Australia).
3.7.3 Serum

Mice were bled either via the tail vein or via the retroorbital route. The blood was allowed to clot before being centrifuged at 13,000rpm in a microfuge for 10 min. Serum was pipetted off and stored at -20°C until use.

3.7.4 Serum TRF Assays

Mouse serum was assessed for specific immunoglobulins via a TRF assay. Briefly, Maxisorp plates (Nunc) were coated with 1μg/ml of the specific antigen as well as a control protein. Plates were blocked with TRF blocking buffer, washed and serum added. The serum was serially diluted in assay buffer (Nunc), and allowed to react for 2 hours at RT or overnight at 4°C. Bound antibodies were detected with a biotinylated anti-mouse Ig monoclonal antibody (Amersham Pharmacia) and streptavidin-europium (Nunc). Enhancement solution was allowed to react and the plate read on the Wallac Victor 2 counter. A significant titre was defined as being higher than two standard deviations above the mean of normal mouse serum.

For competition assays, the protocol was as above, except that a competing protein or antibody was added at the same time as the serum.

3.7.5 Bronchoalveolar Lavage

For lavage, animals were euthanised with CO₂ and an incision made between the cartilaginous rings below the mandible. A cannula constructed from 2cm of 1mm polyethylene tubing attached to a 23G needle was inserted into the incision, and used to lavage the lungs with 3x300μl of PBS + 0.2% BSA. Cytospin preparations of 5 x 10⁴ cells were made using a cytocentrifuge and the cells allowed to dry for 30min before
staining with Diff Quik (Lab Aids, Narrabeen, Australia) as per the manufacturer’s instructions.

3.7.6 Passive Cutaneous Anaphylaxis

Passive cutaneous anaphylaxis (PCA) was used to determine IgE levels of mouse serum. WAG rats were anaesthetised with 0.5ml/100g body weight of 5.7% chloral hydrate (Rampie, Welshpool, WA, Australia) via the intraperitoneal route. Mouse sera was diluted initially four-fold, and doubling dilutions were performed; 50μL of each dilution was injected into the rats intradermally. After twenty-four hours, the rats were challenged intravenously with 10mg of spent mite medium (SMM) or recombinant Der p 2 in 0.5% Evans blue (ICN Biomedicals Inc., Ohio, USA) in saline. The reciprocal of the highest serum dilution giving a blue spot was taken as the PCA titre.

3.8 STATISTICAL ANALYSIS

All statistics were performed using Prism graph software (version 4.0b, Graphpad Software Inc.). Data points were expressed as mean ± standard error (SE) unless otherwise indicated. Comparison of sample means was made using the Mann-Whitney test, with significance at a probability value of <0.05.
CHAPTER FOUR

BLOCKING ABILITY OF ANTI-PAPAIN MONOClonAL ANTIBODIES
4.1 INTRODUCTION

One of the major goals of allergen immunotherapy or vaccination, and the basis for the potential application of mimotopes as immunotherapeutic agents, is the development of IgG blocking antibodies. Though the role of allergen-specific IgG generated by immunotherapy has been controversial, the development of IgG1 and IgG4 during allergen immunotherapy has been well characterised, and is currently generally accepted to be one of the central mechanisms of immunotherapy.

Antibodies of the IgG subclasses raised during immunotherapy have been demonstrated to possess the ability to block the binding of IgE-allergen complexes to mast cells (Nouri-Aria at al., 2004), as well as the ability to inhibit histamine release from basophils (Mothes et al., 2003). The ability of mouse IgG1 antibodies to block the interaction of human IgE and allergen as well as allergen-induced basophil degranulation has also been demonstrated (Vrtala et al., 1998).

It is known that antibodies can either enhance or repress the allergic response. Sehra et al. (2003) administered IgG to ovalbumin sensitised mice before challenging the animals with ovalbumin. The mice who had received the antibody demonstrated reduced eosinophilia and decreased goblet cell metaplasia, as well as a skewing of the immune response towards a Th1 dominant response. In this case, suppression was independent of antibody isotype, with both IgG1 and IgG2a repressing the allergic response. Suppression was dependent on the development of antibody-allergen complexes. Allergen-specific IgG1 has also been implicated as an enhancer of the allergic response by binding to FcγRIII on murine mast cells and activating the cells (Macedo-Soares et al., 2004).

Papain was selected as a model allergen for the investigation of the potential blocking ability of murine antibodies for several reasons. The protein is commercially available at a high level of purity; many of the recombinant allergens that could have been utilised require many days work in order for sufficient amounts to be produced at a high purity. The antigenicity of papain has been previously well established, and a model of allergen sensitisation developed using papain. The protein also has the advantage of
being a homolog of the major house dust mite allergen Der p 1, as well as an allergen in its own right.

To date, no monoclonal antibodies directed against papain have been described. This chapter describes the first known monoclonal antibodies directed against papain, and the examination of these antibodies for blocking ability in an established mouse model of allergic sensitisation.
4.2 RESULTS

4.2.1 Papain Monoclonal Antibodies

Monoclonal antibodies directed against papain were produced from C57BL/6J mice. Animals were immunized with papain emulsified in Freund’s adjuvant and their sera assessed for the presence of anti-papain antibodies before being sacrificed and spleen cells harvested. All animals produced a high titre of anti-papain antibodies.

Four anti-papain mAbs were produced, designated 10A3, 3D5, 8E11 and 9B5, all of which were of the IgG1 isotype.

4.2.1 Effects of monoclonal antibody administered before allergen sensitisation

Groups of five C57BL/6J mice were administered 500 μg of antibody via the intravenous route, either as a single antibody or a combination of antibodies to a total of 500 μg, before being sensitised on the same day with an intranasal dose of 1 μg papain. Intranasal administration of papain was repeated on the subsequent four days. The animals were boosted again with five daily intranasal doses of papain after two weeks, and then challenged after a further two weeks. The challenge consisted of three daily intranasal doses of 100 μg papain. After 24 hours, mice were sacrificed and a bronchoalveolar lavage (BAL) completed.

The IgE titres from each group of mice are shown in figure 4.1. Overall, there were no significant differences in IgE between the groups, indicating that the administration of the mAbs before sensitisation had no effect on IgE production.

The effects of mAb before sensitisation on the cell numbers in the BAL are shown in figure 4.2. Significant differences were seen in the following groups. Total cell numbers were reduced in the mAb mix group, while reduced macrophages were seen in the mix and 9B5 group, reduced lymphocytes in the 9B5 and mix group, and reduced eosinophils in the 8E11 and mix group. No significant differences were seen in
neutrophil numbers. It should be noted that the differences in eosinophils between the 8E11 and 10B2 groups, and 8E11 and mAb mix group approached significance. For each of the cell types where a single mAb and mix resulted in decreases, the decrease was similar between the single mAb and mix, indicating that the single mAb in the mix could have been responsible for the decrease in the mix group.

4.2.2 Effect of administration of monoclonal antibody at time of allergen challenge

The same protocol was followed as for section 4.2.1, except that the mAb dose was given on the first day of the 100μg papain challenge, rather than on the first day of sensitisation. The effect on cell types in BAL is shown in figure 4.3. A very promising result was the decrease in the number of eosinophils by 8E11 and 3D5. In this case, the mAb mix had no effect.

4.2.3 Bromelain controls

The same experiments as in sections 4.2.1 and 4.2.2 were repeated, substituting bromelain for papain. Results of giving the mAb before sensitisation are shown in figure 4.4. Administration of the anti-papain antibodies did not significantly reduce any of the responses to bromelain. The administration of 8E11, to the contrary, resulted in a small (25%) increase in lymphocytes. When the mAbs were given before challenge, there was no significant effect (figure 4.5).
4.3 DISCUSSION

Allergen immunotherapy results in the generation of IgG antibodies potentially capable of blocking allergen-IgE interactions (reviewed in Flicker and Valenta, 2003). The precise role of these antibodies has been controversial, but a number of recent studies have demonstrated that they can possess the ability to inhibit the binding of allergen-IgE complexes to mast cells (Nouri-Aria et al., 2004), inhibit histamine release from basophils (Mothes et al., 2003) and inhibit allergen presentation to T cells (van Neervan et al., 1999). Most of these studies have examined the effect of blocking antibodies in vitro, and none of them examined the effect of blocking antibodies upon allergen sensitisation as a whole. The production of regulatory or blocking antibodies by mimotope administration is also one of the central processes in successful mimotope immunisation. The work described in this chapter examined the effect of potential blocking antibodies in vivo in the context of an animal model of allergic sensitisation.

Four different anti-papain monoclonal antibodies were produced by standard methods. All of the mAbs were of the IgG1 isotype. It has been previously reported that enzymatically active papain preferentially induces an IgG1 response in mice (Chambers et al., 1998), so this could be considered. Experimentally however, an IgG2a response to papain was observed in the mice used for the fusions, though repeated efforts to produce a mAb of the IgG2a isotype failed.

The potential blocking ability of the mAbs was first examined by administering them to mice before sensitisation. None of the antibodies had a significant effect on IgE production, indicating that they were not capable of directly inhibiting IgE production. However, when the mice were challenged and a BAL performed, significant differences were observed in cellular infiltration into the lung.

A significant decrease in the total cell number as well as numbers of macrophages, lymphocytes and eosinophils was seen in the group treated with a mixture of all four mAbs, indicating that the four mAbs acting in concert were able to suppress the immune response. Individual mAbs could also induce effects, so that the effect of the mAb mix was the same as one of the mAbs alone, indicating that the effect could have been the result of a single antibody within the mix.
The mAb 9B5 resulted in significant decreases in the number of both macrophages and lymphocytes. The decrease in macrophages is interesting because macrophages possess the ability to suppress airway hyperresponsiveness as well as acting as antigen presenting cells (Peters-Golden, 2004). The reduction of macrophage numbers could therefore result in an increase in airway hyperresponsiveness. The decrease in lymphocytes is indicative of a suppressed immune response, indicating that this mAb is acting to suppress cellular infiltration into the lungs. Despite the decreases in lymphocytes and macrophages, the number of eosinophils was unchanged, indicating that this mAb was capable of reducing inflammation, but not of affecting the final allergic response.

Administration of the mAb 8E11 resulted in a significant decrease in eosinophil numbers, though other cell types were unaffected. This result indicates that this mAb was acting directly to inhibit allergen sensitisation, though it had no effect on lung inflammation with respect to the other cell types. The 8E11 results, along with the 9B5 results, are extremely significant results, providing evidence that antibodies directed against an allergen can be capable of directly inhibiting allergen sensitisation and inflammation when administered prophylactically.

The antibodies were also examined for their effect on mice previously sensitised to allergen. Administration of the mAbs 8E11 and 3D5 both resulted a significant decrease in the number eosinophils, indicating that they possessed a direct blocking effect on the allergic reaction. This result is again extremely significant, demonstrating that antibodies can be capable of inhibiting the allergic reaction even after allergen sensitisation.

None of the mAbs had any inhibiting effect on sensitisation to bromelain, although 8E11 causes a small increase in lymphocytes in the BAL when delivered before sensitisation. The blocking effects of these antibodies were therefore antigen specific.

The lack of effect on allergen-specific IgE levels was not unexpected. It has been previously reported that successful allergen immunotherapy is not always associated with a significant decrease in IgE levels, but is rather associated with an increased in allergen-specific IgG and a reduction in the mucosal recruitment of inflammatory cells
(Wilson et al., 2001). The experiments performed in this chapter essentially mimicked the effect of allergen immunotherapy by artificially increasing the amount of allergen-specific IgG present. Three of the mAbs tested, 8E11, 9B5 and 3D5, were capable of inhibiting inflammatory cell infiltration into the lungs in the same fashion as successful immunotherapy. The ability of the mAbs 8E11 and 3D5 to decrease eosinophilia after established allergic sensitisation is particularly significant, as it demonstrates the ability of antibodies to directly inhibit the allergic reaction in a previously sensitised individual.

The reduction in cellular infiltration observed with antibody administration before sensitisation indicates that the antibodies are capable of exerting specific inhibitory effects on different aspects of the allergic response. At challenge, decreases in the numbers of macrophages, lymphocytes and eosinophils were observed. The inhibition of cellular infiltration could not have been due to blocking antibodies remaining in the serum, as the half life of IgG1 in murine serum is only 6-8 days (Vieria and Rajewsky, 1988). The inhibition of allergic sensitisation was therefore inhibiting some of the later allergic response.

The different activities of the antibodies could potentially be attributed to differences in their epitopes on papain. Vrtala et al., (1998) demonstrated that murine anti-allergen IgG1 antibodies that recognised the same epitope as human IgE were capable of inhibiting the interaction of human IgE and allergen, as well as inhibiting allergen-induced human basophil degranulation. It has been reported previously that antibodies directed against Bet v 1 have differing biological activity (in terms of enhancing or inhibiting IgE binding and specific histamine release); the activity of the antibody depended on its epitope specificity (Lebecque et al., 1997). It is possible that a similar situation exists for the anti-papain antibodies.

Two different classes of IgG1 antibodies have been described: anaphylactic and nonanaphylactic IgG1. Anaphylactic IgG1 enhances lung eosinophilia and airway hyperresponsiveness (Macedo-Soares et al., 2004). Production of these classes of antibodies is adjuvant dependent; the use of CFA, as in the production of the anti-papain mAbs in this study results mainly in nonanaphylactic IgG1 production (Faquim-Mauro and Macedo, 2000).
The effect of IgG administration to ovalbumin sensitised mice before antigen challenge has been investigated by Sehra et al (2003). The antibody was administered intranasally, and resulted in decreased cellular infiltration into the lungs, including a decrease in eosinophilia. The effect was allergen specific; unrelated allergen antibodies had no effect on the ovalbumin response, and isotype independent. The examination of isotype effect was not possible in the current work, as all mAbs isolated were of the IgG1 isotype and attempts to produce a IgG2a mAb directed against papain were unsuccessful (results not shown).

A potential mechanism for the action of blocking antibodies is the blocking of IgE binding to allergen, thus preventing cross-linking of mast cell-bound IgE and subsequent degranulation of mast cells. It is known that histamine released from mast cells induced endothelial cells to produce mediators including eotaxin, a potent eosinophil chemoattractant (Brightling et al., 2003; Menzies-Gow et al., 2004); as such, a decrease in mast cell degranulation would be expected to result in a decrease in eosinophil infiltration, as was observed with the mAbs 8E11 and 3D5. However, it is known that mast cell deficient mice can still demonstrate eosinophilia (Takeda et al., 1997), indicating that mast cell degranulation is not the only mechanism acting to stimulate eosinophil infiltration into the lungs, and therefore may not be the only target for blocking antibodies.

It has also been previously demonstrated that blocking antibodies are able to inhibit allergen specific T cell activation by inhibiting antigen presentation (van Neervan et al., 1999). Interleukin-5 produced by activated T cells induces eosinophilopoiesis in the bone marrow (Wardlaw, 2004). The prevention of activation of T cells by blocking antibodies would therefore be expected to reduce total numbers of eosinophils. It is possible that inhibition of T cell activation was one mechanism of action of the blocking anti-papain mAbs.

In summary, three different anti-papain mAbs have been shown to have a blocking effect on papain sensitisation and challenge in a murine model. The specific effects of the mAbs varied, though they were restricted to changes in cellular infiltration into the lungs; anti-papain IgE levels were not affected. Some mAbs had no inhibitory activity in any of the assay systems. The blocking effect was antigen specific, and an unrelated mAb did not have any blocking effect on papain sensitisation. Further elucidation of the
actions of these antibodies could be achieved by an examination of the cytokine profile of the BAL fluid or by examination of airway hyperresponsiveness.
Figure 4.1 IgE titres; monoclonal antibodies administered before sensitisation.

Groups of five mice were given 500μg of the monoclonal antibodies via the intravenous route on the morning of the first day of sensitisation. IgE titres were measured via PCA. PBS was used as a negative control, the 10B2 monoclonal antibody (directed against Der p 2) was also a negative control. Results show mean ± standard error (SE).
Figure 4.2  Cell counts of BAL fluid; antibody administered before sensitisation.

Groups of five mice were given 500μg of the monoclonal antibodies via the intravenous route on the morning of the first day of sensitisation. PBS and 10B2 were negative controls. Results show mean ± standard error (SE).
Figure 4.3 Cell counts of BAL fluid; antibody administered at challenge.

Groups of five mice were given 500μg of the monoclonal antibodies via the intravenous route on the morning of the day of antigen challenge. PBS and 10B2 were negative controls. Results show mean ± standard error (SE).
Figure 4.4 Cell counts of BAL fluid; antibody administered before sensitisation, bromelain control.

Groups of five mice were given 500μg of the monoclonal antibodies via the intravenous route on the morning of the first day of sensitisation. PBS and 10B2 were negative controls. Results show mean ± standard error (SE).
Groups of five mice were given 500μg of the monoclonal antibodies via the intravenous route on the morning of the first day of sensitisation. PBS and 10B2 were negative controls. Results show mean ± standard error (SE).
CHAPTER FIVE

PAPAIN MIMOTOPES
5.1 INTRODUCTION

Papain was adopted as a model protein for the investigation into the isolation and utility of allergen mimotopes for several reasons. As well as being an occupational allergen in its own right (Baur et al., 1982), papain is a homologue of the major house dust mite allergen Der p 1. Unlike Der p 1, papain is available commercially at high purity. The protein has been fully sequenced and its three-dimensional structure resolved. As described in chapter four, anti-papain mAbs with a range of biological activities have been produced, facilitating the potential isolation of a range of papain mimotopes. Papain has also been utilized in a murine model of asthma (Lenzo et al., 2004), facilitating the possibility of examining the application of mimotopes in this model.

The three dimensional structure of papain has been resolved by X-ray crystallography (Drenth et al., 1968; Kamphus et al., 1984; Yamamoto et al., 1991). Comparison of the structure of papain with the modeled structure of Der p 1 and other cysteine proteases revealed a common structural motif shared between the proteins. This motif was located in a groove between an α-helix and a β-sheet and contains the active site of Der p 1 and papain (Furmonaviciene and Shakib, 2001).

Furmonaviciene et al. (2000) utilized the three-dimensional models of cysteine proteases including Der p 1 and papain to investigate the presence of a putative cross-reactive IgE epitope. Previous work by the authors using phage display libraries had identified residues Leu147-Gln160 as a human IgE binding region of Der p 1 (Furmonaviciene et al., 1999). The location of this epitope was identified on the three-dimensional models of the other cysteine proteases; the shape and theoretical degree of antibody accessibility varied between the proteins. The presence of this epitope on papain was confirmed by the use of a mAb cross-reactive between papain and Der p 1. To date, this is the only study that has investigated the nature of papain epitopes, and it was limited to in vitro work.

This chapter describes the use of papain specific mAbs as targets for biopanning of random peptide phage display libraries and the characterization of potential papain mimotopes. It was reasoned that if mimotopes could be obtained they would provide an excellent resource for studying their ability to modify allergen responses.
5.2 RESULTS

5.2.1 Biopanning

Biopanning on all of the anti-papain mAbs was performed with the combined PhD libraries and the f884 library. In each case, \(10^{11}\) pfu was used as the input for the first round of biopanning.

The enrichment of phage over rounds of biopanning is shown in Table 5.1. Phage enrichment was observed with the biopanning of the combined PhD libraries on the mAb 10A3. The lack of enrichment seen with biopanning of the other libraries precluded further investigation.

5.2.2 ELISA Screening of Clones

Individual clones from the fourth round of PhD biopanning on 10A3 were purified and amplified on a small scale before being screened in an ELISA as per section 3.1.6 (figure 5.1). A total of 20 clones were screened against 10A3 and an isotype control antibody. Nine clones exhibiting higher binding to 10A3 than the control antibody were selected for further analysis.

5.2.3 Competition ELISA

Nine clones, designated 10A3P1-9 were analysed via a competition ELISA. Individual phage clones were amplified and titred before use in this assay. Equivalent titres of phage were used per well, with 5ng of papain or ovalbumin added to duplicate wells. The percentage inhibition by papain is shown in Table 5.2; none of the clones were inhibited by ovalbumin. Inhibition by papain ranged from 30.5 to 100%; clones 10A3P1 (inhibited 100%) and 10A3P3 (inhibited 99%) were selected for immunological analysis.
5.2.4 Sequencing

Single stranded DNA was isolated from all nine of the 10A3 specific phage clones and sequenced by use of the –96 primer. The peptides displayed by these phage are shown in table 5.2. Each of the sequences was isolated once only, and no overall similarity was seen between all of the peptides when considered as a single group. However, there was similarity when the peptides were stratified into two groups, as shown in figure 5.2. No similarity to the papain primary sequence was observed for any of the clones.

The clones 10A3P1, 2 and 6 displayed similar peptides. These three clones were competed in a similar fashion by papain for 10A3 binding. Further similarity was also seen between clones 10A3P1 and 4, though this did not correlate with papain competition.

5.2.5 Immunisation

Groups of three C57BL6/J mice were immunized by repeated injection with approximately $10^{11}$pfu of phage clone 10A3P1 or 10A3P3 via the intraperitoneal route; no adjuvant was used. Serum was assessed for antibodies directed against papain and a control antigen (table 5.3); no specific papain antibodies were seen. This experiment was repeated several times with the same result; while high titres of anti-phage antibodies were consistently produced, no detectable amount of anti-papain antibodies were ever produced.
5.3 DISCUSSION

Four different papain monoclonal antibodies were used to biopan the PhD and f884 random peptide phage display libraries. Successful enrichment was observed when the combined PhD libraries were biopanned against the mAb 10A3. Repeated attempts at biopanning were necessary to produce this enrichment. Individual clones were analysed from the other biopanning experiments with the other mAbs (results not shown); none of these phage clones displayed specificity in an ELISA assay for the selecting mAb. It is probable that the libraries used did not contain specific binding peptides for these antibodies, or that specific peptides were extremely rare in the libraries, and lost during the biopanning procedure. Failure of biopanning to isolate specific peptides is not uncommon; it has been reported that only one half of targets yield a specific peptide (Kay et al., 2001). It was hoped that mimotopes would be isolated with one of the biologically active mAbs, as described in chapter four. Unfortunately, the only antibody that resulted in the isolation of specific clones (10A3) was the only antibody that possessed no blocking activity.

Analysis of the individual phage clones from the fourth round of biopanning of the PhD libraries on 10A3 revealed that approximately half of the isolated clones reacted specifically with 10A3 in a TRF assay. The non-specific clones were assumed to represent non-specific plastic or immunoglobulin binders and were discarded. The isolation of phage clones specific for all components of the biopanning procedure, including plastic, have been well documented, and does not indicate failure of biopanning (Adey et al., 1995).

Nine 10A3 specific clones were investigated further in a competition assay; all of the clones were competed specifically by papain, indicating that all of the displayed peptides mimicked the 10A3 binding epitope of papain. Sequencing of the phage clones revealed that they all displayed different peptides. Overall, there was no sequence similarity between them as a group, however subsets of clones displayed similarities that represented a clear consensus sequence.

The mimotope selected by Furmonoviciene et al. (1999) using an antibody cross-reactive between papain and Der p 1 showed no similarity to any of the peptides selected with 10A3. Comparison of the 10A3 selected peptides with peptides selected
by other monoclonal antibodies revealed no similarity, indicating that these peptides are indeed antibody specific. There were no significant similarities observed between the 10A3 peptides and papain or Der p 1.

The two clones displaying the highest binding to 10A3 and competition by papain were selected for immunisation trials in C57BL6/J mice. This strain was selected because it is known to respond to papain, and is the strain employed in the papain model of allergic sensitisation. As filamentous phage are highly immunogenic and possess inherent adjuvant activity (Frenkel et al., 2000), the trials were carried out without the use of an adjuvant. None of the mice immunised developed a significant titre of anti-papain antibodies even after four boosts.

While it has been reported that peptides selected by biopanning are not necessarily immunogenic, as described for pertussis toxin mimics (Felici et al., 1993). It is also possible that the immunisation protocol utilised was not optimal for the development of anti-mimotope antibodies. The PhD phage display libraries in particular display only five copies of the recombinant peptide per virion, resulting in only a small amount of peptide being delivered to the mouse.

Previous studies examining the immunogenicity of phage-displayed peptides have generally demonstrated that pIII displayed peptides are less immunogenic than pVIII displayed peptides, probably due to differences in copy number. Yip et al. (2001) demonstrated the immunogenicity of a mimotope of Erb-2 in both pVIII and pIII display vectors. Immunisation with pIII required five boosts to achieve the same antibody titre seen with pVIII immunisation after three boosts. All of these experiments used Freund’s adjuvant. Successful immunisation with pIII displayed hepatitis B mimotopes without the use of adjuvant has been achieved, though the same mimotope displayed on pVIII resulted in a higher and more reproducible response (Meola et al., 1995). Mimotopes of Alzheimer’s β-amyloid proteins have been used to immunise mice in pIII and pVIII display; the pVIII vector was highly immunogenic, while the pIII vector was only weakly immunogenic (Frenkel et al., 2000).

The results presented in this chapter as well as he above studies suggest that the immunisation protocols used were not optimal. In particular, for pIII displayed peptides, adjuvant may be required, as will be studied in later chapters.
Table 5.1 Biopanning of phage display libraries with anti-papain monoclonal antibodies.

The four different monoclonal antibodies, 10A3, 3D5, 9B5 and 8E11, were used to biopan the combined PhD and f884 libraries. The percentage recovery of phage for rounds 1 to 4 was calculated by dividing the output titre by the input titre. A significant increase in phage recovery is defined as a greater than hundredfold increase.

<table>
<thead>
<tr>
<th></th>
<th>10A3</th>
<th>3D5</th>
<th>9B5</th>
<th>8E11</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhD Panning</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>0.009</td>
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<td>0.0009</td>
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</tr>
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<td>0.006</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>3</td>
<td>0.003</td>
<td>0.002</td>
<td>0.02</td>
<td>0.003</td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>0.06</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>F884 Panning</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>0.006</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
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<td>0.0005</td>
<td>0.0002</td>
</tr>
<tr>
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<td>0.003</td>
<td>0.0025</td>
<td>0.003</td>
</tr>
<tr>
<td>4</td>
<td>0.002</td>
<td>0.003</td>
<td>0.003</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Figure 5.1 ELISA of round four PhD 10A3 clones.

Representative data from a single biopanning experiment. Phage clones were purified and titred before being tested for reactivity against 10A3, the isotype control antibody 7A1 and blocked plastic. Clones exhibiting higher binding to 10A3 than the two controls were selected for further investigation.
Table 5.2 PhD 10A3 clones.

All of the phage clones were isolated only once. The ELISA reactivity is the optical density at 630nm of the phage when tested on solid phase 10A3. The percentage inhibition is for the addition of 5ng of papain to phage.

<table>
<thead>
<tr>
<th>Mimotope</th>
<th>Sequence</th>
<th>ELISA Reactivity</th>
<th>% Inhibition by Papain</th>
</tr>
</thead>
<tbody>
<tr>
<td>10A3P1</td>
<td>WAPPIYTTPYP</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td>10A3P2</td>
<td>KAPHLFRCCNMS</td>
<td>0.6</td>
<td>78.6</td>
</tr>
<tr>
<td>10A3P3</td>
<td>NFMECLPRLMGH</td>
<td>0.9</td>
<td>99</td>
</tr>
<tr>
<td>10A3P4</td>
<td>HMFSYAHVPYVV</td>
<td>0.6</td>
<td>40.7</td>
</tr>
<tr>
<td>10A3P5</td>
<td>KHLNPFLEGRTF</td>
<td>0.3</td>
<td>30.5</td>
</tr>
<tr>
<td>10A3P6</td>
<td>KAPQLFVQPMS</td>
<td>0.17</td>
<td>76.7</td>
</tr>
<tr>
<td>10A3P7</td>
<td>MDKMTTNVIKT</td>
<td>0.3</td>
<td>49.3</td>
</tr>
<tr>
<td>10A3P8</td>
<td>YTKPMGLTFPSL</td>
<td>0.22</td>
<td>48</td>
</tr>
<tr>
<td>10A3P9</td>
<td>KHMHHWHPPALNT</td>
<td>0.1</td>
<td>55.7</td>
</tr>
</tbody>
</table>
Figure 5.2 ClustalW alignment of PhD 10A3 sequences.

Panel A shows the three most similar sequences; all of these were competed similarly by papain. Panel B shows the alignment of the 10A3P1 and 4 sequences, illustrating the “PY” motif. Panel C shows a dendrogram of the peptide sequences.
Table 5.3  Mouse immunisation with PhD 10A3 phage.

The titres given are the last dilution to give a significantly higher response than normal mouse serum for papain and a control allergen, Bet v 1.

<table>
<thead>
<tr>
<th>Mimotope</th>
<th>Sequence</th>
<th>Papain titre $(-\log^{10})$</th>
<th>Bet v 1 titre $(-\log^{10})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10A3P1</td>
<td>WAPPIYTKTPYP</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10A3P3</td>
<td>NFMECLPRLGMH</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
CHAPTER SIX

FLAG: A MODEL EPITOPE
6.1 INTRODUCTION

The FLAG peptide, DYKDDDDK, is a synthetic tag that was developed and used primarily for the purification of fusion proteins. The success of the FLAG peptide as an affinity tag has been attributed to the fact that it does not interfere with the native folding of its fusion partner, as well as the facts that the peptide itself is immunogenic and water-soluble and retains a high degree of exposure on the surface of the fusion protein (Einhauer and Jungbauer, 2001).

Three monoclonal antibodies specific for the FLAG peptide are commercially available, all of which were originally raised in BALB/c mice (Einhauer and Jungbauer, 2001). These antibodies are designated FLAG M1, M2 and M5. While they all recognise the FLAG epitope, they vary in their ability to recognise the peptide at different positions on the fusion peptide, as well as their requirement for Ca$^{2+}$ for FLAG peptide binding. The FLAG M2 mAb is the most versatile, being able to bind to the FLAG peptide as an amino or carboxyl terminal fusion, as well as an internal part of the fusion protein. This antibody does not require calcium for binding. The FLAG mAbs all display high affinity for the FLAG peptide.

Sloostra et al. (1996) examined the interaction of the FLAG mAbs with a series of variants of the FLAG peptide by performing ELISAs on synthetic peptides representing the variants. Several different variants of the FLAG peptide were identified that were able to bind to the mAbs, some with greater affinity than the canonical FLAG peptide. For the M2 mAb, the sequence MDYKAFDNL was shown to display greater sensitivity in an ELISA than the native FLAG sequence when adsorbed onto a solid surface. However, when the peptide variant was used in solution, it displayed lower binding than the native sequence, indicating that it did not possess a rigid structure when free in solution. Overall, when the binding properties of many FLAG variants were compared, the authors concluded that residues Y2, K3, and to a lesser extent, D1 and D6 were critical for the binding of the M2 mAb.

The FLAG M2 mAb was used as a target for biopanning of phage display libraries by Miceli et al. (1994). The mAb was used to screen a random dodecapeptide protein III display library. Three residues were conserved across binding clones, representing the
sequence YKXXD, where X represented a range of amino acids. The authors went on to construct a library based on this sequence, randomising the residues other than those identified as critical for binding. Biopanning on this library revealed a consensus sequence of DYKXXD, corresponding to D1, Y2, K3 and D6 of the FLAG epitope. Nuclear magnetic resonance (NMR) spectroscopy of a peptide representing this consensus sequence revealed that D1 and D6 were the residues most likely to interact with the Fab fragment of the M2 mAb (Stockman et al., 1995).

Due to the equivocal nature of the mimotopes isolated with the anti-Der p 1 (appendix one) and anti-papain (chapter five) mAbs, the FLAG system was adopted as a model system for the investigation of mimotopes. The fact that the FLAG peptide is immunogenic was an important consideration. The peptide could be used to examine immunisation protocols and if necessary could be used to examine the effect of anti-epitope antibodies using allergens genetically engineered to contain the FLAG epitope.

This chapter describes the biopanning of three different phage display libraries using the FLAG M2 mAb as a target, and the investigation of the potential immunogenicity of the isolated FLAG clones.
6.2 RESULTS

6.2.1 Biopanning against anti-FLAG M2 monoclonal antibody

Four phage display libraries were biopanned on the FLAG M2 mAb. The libraries used were the pIII random display filamentous PhD12 and PhD7 libraries, the random pVIII filamentous f884 library, and the T7 phylomer library. Significant enrichment (or enrichment approaching significance) of phage at round three or four were observed with all libraries except PhD12, as shown in table 6.1.

6.2.2 Analysis of FLAG M2 PhD12 clones

Individual phage clones from round three of biopanning with the PhD12 library were purified and analysed in a TRF assay and a competition assay, the results of which are shown in figure 6.1. An unselected aliquot of the library was unreactive with either antibody and no increase in reactivity of pools of phage eluates to the FLAG M2 mAb was seen over the rounds of panning. Of ten clones analysed, nine exhibited higher binding to the FLAG M2 mAb than to the isotype control mAb.

Nine specific clones were examined in a competition assay; the results of competing with the FLAG peptide are shown in figure 6.1. None of the clones were specifically competed by the FLAG peptide, indicating that they probably represented nonspecific immunoglobulin binding or very low affinity phage. No further experiments were therefore performed using these clones.

6.2.3 Analysis of FLAG M2 PhD7 clones

Twenty phage clones from the third round of PhD7 biopanning were purified and analysed via TRF and competition assays (figure 6.2). All of the clones exhibited higher binding to FLAG M2 than to an isotype control, while the control M13K07
phage did not bind to either antibody. Five clones – clones 1, 2, 7, 9 and 17 – were specifically competed to varying degrees by the FLAG peptide, and hence were concluded to be binding specifically to the FLAG M2 mAb.

The sequence of the peptide displayed by these phage clones was inferred from ssDNA sequencing with the –96 sequencing primer. A ClustalW alignment with the canonical FLAG peptide was carried out (figure 6.3). A conserved sequence, YQ - - Y was observed between the phage displayed peptides, with only the initial tyrosine common to both the phage clones and the FLAG peptide.

6.2.4 Analysis of FLAG f884 clones

Thirty clones from the fourth round of f884 biopanning were purified and analysed via TRF and competition assays, the results of which are shown in figure 6.4. All of the phage clones exhibited an extremely high level of binding to the FLAG M2 mAb and low binding to the isotype control. Competition analysis of ten of these clones showed that all were competed specifically in a dose-dependent fashion by the FLAG peptide.

The peptide displayed by four of the specific phage clones was determined by sequencing of the phage genome with the f884 sequencing primer. The displayed peptides were aligned with the FLAG peptide (figure 6.5); a consensus sequence, DYK - - D, was observed between three of the phage clones and the FLAG peptide. The fourth clone shared the consensus YKD – D with the FLAG peptide.

6.2.5 Analysis of T7 phylomer FLAG clones

Thirteen clones from the third round of biopanning of the T7 phylomer library on the FLAG M2 mAb were purified and analysed via TRF and competition assays, the results of which are shown in figure 6.6. Eleven of the clones displayed higher binding to FLAG M2 than to an isotype control. A slight increase in FLAG M2 binding was observed over the rounds of biopanning, while wild type T7 did not bind significantly. Nine clones were competed specifically by the FLAG peptide, and were inferred to be binding specifically to FLAG M2.
The peptides displayed by these clones were determined by sequencing of phage DNA with the T7 reverse primer. These peptides were aligned (figure 6.7); none of these clones shared more than two residues in common with the FLAG peptide. Clone 3.7 shared the sequence D - - - - - - K, while clone 3.11 shared - - K - - - - K. Three of the clones (clones 5, 12 and 13) also displayed similarity at the beginning of the displayed peptide.

The origin of the selected peptides was determined by comparing the sequences to the GenBank database (table 6.2). Two of the clones contained inserts clearly originating from two of the organisms used to generate the library. The origin of the other clones could not be determined; they probably originated from poorly annotated or variable regions within the genomes used, or from cloning artifacts.

6.2.6 Immunisation experiments with the FLAG peptides and mimetics

Two phage clones from each library were selected for use in pilot immunisation experiments, the results of which are shown as TRF counts with error bars in figure 6.8. Groups of three mice were immunized with approximately $10^{11}$pfu of phage with CFA via the subcutaneous route. Mice were boosted twice at monthly interval, substituting IFA for CFA, and bled 7-10 days after the final dose. Only one clone, f884 4.1, resulted in the generation of antibodies capable of binding to the FLAG peptide, though only two mice out of a group of three responded. The peptide displayed by this clone (figure 6.5) was very similar to the canonical FLAG sequence, sharing the residues DYK - - D.

Further immunization experiments were performed using the canonical FLAG peptide cloned into the T7-415 vector. Phage were pretreated with SDS to denature the virion into its component proteins, as this had resulted in more consistent results in other experiments (chapter seven). SDS itself is also known to act as an adjuvant (Clausen et al., 2000). Groups of five mice, either C57BL6/J, BALB/c or CBA, were immunized via the subcutaneous route with approximately $10^{11}$pfu of T7-FLAG pretreated with SDS and emulsified in CFA. Mice were boosted twice at monthly interval, substituting IFA for CFA, and bled 7-10 days after the final dose. Specific anti-FLAG antibodies were produced, with all strains responding equally well (figure 6.9).
The FLAG peptide was also cloned as a GST fusion protein and used to immunize mice. A group of five C57BL6/J mice was immunized with GST-FLAG emulsified in CFA via the subcutaneous route, and boosted twice at monthly intervals, substituting IFA, and bled 7-10 days after the final dose. No specific anti-FLAG antibodies were produced (results not shown).
6.3 DISCUSSION

The FLAG peptide was employed as a model epitope for the examination of phage display library biopanning. Four different phage display libraries were biopanned against the FLAG M2 mAb. This antibody was chosen because it is capable of binding the FLAG peptide as both an amino- and carboxyl-terminal fusion, allowing the screening of both the M13 and PhD phage display libraries with the same antibody. Significant enrichment of phage over the rounds of biopanning was observed for three of the four libraries.

The PhD 12 library was the only library that did not result in a significant enrichment of phage over the rounds of biopanning. Clones isolated from the last round of biopanning displayed only slightly higher binding to FLAG M2 than the isotype control, and were not specifically competed by the FLAG peptide. No increase in specific TRF binding was observed over the rounds of biopanning. The clones isolated from the PhD12 library were therefore all concluded to be nonspecific. As the PhD12 library does not contain a complete representation of all possible dodecamer sequences (in order for complete coverage of all dodecamers one would require \(2^{312}\) individual clones), it is likely that this library did not contain any sequences capable of binding FLAG M2, or that these sequences were rare and lost over the rounds of biopanning. In this case, the lack of enrichment correlated with a failure of biopanning to isolate specific clones.

In contrast to the PhD12 biopanning, the PhD7 library resulted in an extremely high enrichment of phage at the third round of biopanning, with 11% of the input titre recovered. When individual clones were analysed, they all displayed high antigenicity. Five clones were identified that bound specifically to the FLAG M2 mAb and were competed by the FLAG peptide. Analysis of the displayed peptides revealed a consensus sequence, YQ - - Y. This consensus shared only the FLAG Y2 residue, one of the residues previously identified as being critical for FLAG binding to the M2 mAb (Sloostra et al., 1996). The K3 residue of FLAG is substituted with glutamine in the phage peptide consensus, a relatively conservative change according to the PAM250 matrix (Benner et al., 1994), which is therefore not likely to alter the conformation of the peptide when compared to the native peptide to a significant degree. The substitution of tyrosine in the phage peptide consensus for the D6 FLAG residue is not conservative, and is therefore more likely to result in a conformation change and
different peptide properties. The results in this chapter demonstrate that this phage-displayed peptide is capable of binding the FLAG-M2 mAb and is competed specifically by the FLAG peptide, indicating that it is a variant of the FLAG sequence \textit{in vitro}. Similar sequences have been isolated previously by Miceli et al. (1994), strengthening the case for these peptides as FLAG variants despite the lack of strong similarity to the canonical FLAG peptide. For this biopanning reaction, therefore, enrichment of phage correlated with the successful isolation of FLAG variant peptides.

Phage clones exhibiting the strongest binding to the FLAG M2 mAb were isolated from the f884 library; all of these clones were also specifically competed by the FLAG peptide. The high degree of antigenicity as well as lack of strong competition probably reflects the multivalent nature of the f884 phage vector. Sequencing of the displayed peptides revealed that most shared the DYK - - D motif, residues that have previously been identified by Miceli et al (1994) as being the most critical for binding to the FLAG M2 mAb. Only one clone, 4.7, did not share the complete motif, instead displaying the conserved residues YKD-D (the phage pVIII protein did not complete this sequence with the initial asparagines seen in the other selected peptides). As with the PhD12 biopanning, enrichment of phage correlated with the isolation of FLAG variant peptides.

Biopanning of the phylomer library resulted in the isolation of 13 specific clones. Sequence analysis of the displayed peptides revealed no strong similarity between them as a group. None of the sequences shared more than two residues in common with the native FLAG sequence. Clone 3.7 shared the sequence D - - - - - K, while clone 3.11 shared - - K - - - - K. The bacterial origin of two of the peptides was ascertained; both represented internal sequences of known proteins. As with the f884 library, enrichment of phage, as measured by percentage recovery, correlated with the isolation of specific clones. It had been hypothesized that mimotopes expressed within natural open reading frames could assume more physical and immunological stability. As shown at least for clone 3.7, this was not the case.

Immunization with six clones resulted in the generation of antibodies cross-reactive against the canonical FLAG peptide with only the f884 clone 4.1. This clone was the most similar to FLAG and contained all of the residues previously demonstrated to be critical for FLAG mAb binding (Sloostra et al., 1996), and therefore could be expected...
to have been the most immunogenic. These experiments were carried out using Freund’s adjuvant, due to the failure of the papain mimotopes to immunize when delivered without adjuvant (chapter five).

While the f884 4.1 clone demonstrated immunogenicity in the pilot immunization experiments, the variation in individual mouse responses gave rise to a large standard error, meaning that statistical significance could not be demonstrated. It was therefore decided to perform further experiments using the canonical FLAG sequence to explore suitable methods of phage immunisation. These experiments were performed with the FLAG peptide in display on the T7-415 vector or with a GST-FLAG fusion protein.

Immunisation with the GST-FLAG fusion did not generate of FLAG specific antibodies, though the fusion protein was antigenic (results not shown). This experiment was carried out only in C57BL6/J mice. This strain is known to respond to the GST protein (Davern et al., 1987), as well as other GST fusions (see chapter seven) and demonstrated a specific FLAG response when immunized with FLAG and FLAG variants in display on phage vectors. Immunisation of other strains of mice with the GST-FLAG fusion protein would clarify if the lack of response to this fusion was in fact strain dependent, but the line of investigation has instead been continued with the 10B2 epitope described in chapter 7.

In comparison to the GST results, the T7-FLAG phage were immunogenic, but consistent results were only obtained when the phage were pretreated with SDS. Three different strains of inbred mice were compared; no significant differences were observed in the antibody responses of C57BL6/J, BALB/c and CBA mice. The use of SDS could therefore be useful for other peptide display immunizations experiments.
<table>
<thead>
<tr>
<th>Round</th>
<th>PhD12</th>
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<th>T7 Phylomer</th>
</tr>
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<tbody>
<tr>
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</tr>
<tr>
<td>Round 4</td>
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</tr>
</tbody>
</table>

**Table 6.1 Enrichment of phage over rounds of biopanning against FLAG M2 mAb.**

The FLAG M2 mAb was used to biopan the PhD12, PhD7, f884 and T7 phylomer libraries. The percentage recovery of phage was calculated by dividing the output titre by the input titre. A significant increase in phage recovery is defined as a greater than hundredfold increase. Where no figures are given, that round of biopanning was not completed.
Figure 6.1 Analysis of PhD12 FLAG phage.

Panel A shows a TRF of clones from third round of panning, as well as an aliquot from the unselected library (PhD12), and aliquots from the rounds of panning (R1, R2 and R3). Panel B shows a FLAG peptide competition TRF of the same clones and an aliquot of unselected library (PhD12).
Figure 6.2 Analysis of PhD7 FLAG phage.

Panel A shows a TRF of clones from the third round of panning, as well as the wild type M13K07 phage. Panel B shows a competition TRF of the same clones, where 1µg, 2µg and 5µg of FLAG peptide was added per well. Ovalbumin did not compete any clones (not shown on graph).
Figure 6.3 ClustalW alignment of PhD7 FLAG M2 clones.

The sequence of the peptides displayed by the phage clones is shown. A ClustalW alignment with the canonical FLAG peptide was performed using MacVector. Identities or unmatched are highlighted in dark grey.
Figure 6.4 Analysis of f884 FLAG M2 phage.

Panel A shows a TRF of clones from the fourth round of panning. Panel B shows a competition TRF of ten clones, where 1μg, 2μg and 5μg of FLAG peptide was added. Ovalbumin did not compete any phage clones.
Figure 6.5 ClustalW alignment of f884 FLAG peptides.

Sequences shown are the displayed peptides, aligned against the canonical FLAG peptide sequence. Identities or unmatched are highlighted in dark grey, while similarities are highlighted in light grey. Panel A shows the alignment of four displayed peptides with FLAG, while panel B shows the alignment of clone seven against FLAG; the phage pIII does not contain the initial aspartic acid residue.
Figure 6.6 Analysis of phylomer FLAG M2 clones.
Panel A shows a TRF of clones from the third round of panning, as well as wild type phage (T7415), and aliquots from the rounds of panning (R1, R2 and R3). Panel B shows a competition TRF of 13 round three clones, where 1µg, 2µg and 3µg of FLAG peptide was added. Ovalbumin did not compete any clones.
Figure 6.7 Sequences of phylomer FLAG clones.

Sequences shown are the displayed peptides, aligned against the canonical FLAG peptide. Identities or unmatched residues are highlighted in dark grey. A dendrogram of the sequences is also shown.
Table 6.2 Phylomer FLAG sequences.

The displayed peptides were sequenced and compared with the GenBank database in order to determine their origin. The peptide length given is the length of the peptide fused to the T7 protein 10; many of the clones contained more DNA after the stop codon of the peptide.
Figure 6.8 FLAG epitope phage immunisation.

Groups of three mice were immunised with the phage clones with adjuvant via the subcutaneous route. Serum was assessed for specific binding to the FLAG peptide. Results show the mean ± standard error of the anti-FLAG peptide titres.
Figure 6.9 FLAG Phage immunisation with T7 phage treated with SDS.

Groups of five mice were immunised with SDS treated T7 phage with adjuvant via the subcutaneous route. Serum was assessed for specific binding to the FLAG peptide by subtracting the titre to a control antigen (ovalbumin) from the titre to the FLAG peptide. Results shown are means ± standard error of the anti-FLAG peptide titre.
CHAPTER SEVEN

Der p 2 MIMOTOPES
7.1 INTRODUCTION

The success of biopanning with the FLAG M2 mAb (chapter six) and immunisation with FLAG variant phage meant that biopanning for a biologically relevant mimotope could be carried out with more confidence. The protein chosen for this analysis was the major house dust mite allergen Der p 2. This protein is an ideal target for the isolation of mimotopes due to the fact that the three-dimensional structure of the protein has been resolved, and the location of several human and murine epitopes mapped. Monoclonal antibodies directed against Der p 2 are available, as well as small amounts of pure natural protein and larger amounts of pure recombinant protein from both \textit{E. coli} and \textit{Pichia pastoris}. The murine response to Der p 2 has been characterised, and human serum from individuals allergic to Der p 2 available.

The three-dimensional structure of Der p 2 has been resolved by the use of nuclear magnetic resonance (Mueller et al., 1997; Mueller et al., 1998) and X-ray crystallography (Derewenda et al., 2002). Mueller et al. (2001) also examined the epitopes recognised by a series of murine monoclonal antibodies, classifying them into three groups based on epitope recognition. Each group of antibodies were able to inhibit binding of other antibodies within the group, though no inhibition of the other two groups was seen. Antibody binding to natural and recombinant Der p 2 (produced in the pET21a vector in \textit{E. coli}) was also compared, with no significant differences in antibody binding to either protein observed, indicating that rDer p 2 could be substituted for nDer p 2 in assays.

Epitopes of Der p 2 recognised by human IgE were examined by Mueller et al. (2001); this study demonstrated that two of the three previously identified by murine mAbs were also human IgE epitopes. The epitope recognised by the mouse mAb 7A1, though not also representing a human IgE epitope, was further defined by the use of alanine point mutants. Only a small number of residues within the previously defined epitope were absolutely required for antibody binding. This evidence strengthens the theory of mimotopes, which holds that epitopes can be minimised to 7-15 amino acids.
This chapter describes the isolation of Der p 2 mimotopes from random peptide display libraries, and the characterisation of these mimotopes both in terms of immunogenicity and antigenicity. These are the first described Der p 2 mimotopes.
7.2 RESULTS

7.2.1 Biopanning Against Anti-Der p 2 Monoclonal Antibody

An anti-Der p 2 mAb, 10B2, was used as the target for biopanning of four random peptide phage display libraries, PhD7, PhDC7C, PhD12 and f884. This antibody was demonstrated to be specific for Der p 2 by TRF screening against Der p 2 and a range of control antigens (results not shown). The enrichment of phage over rounds of biopanning is shown in table 7.1. Enrichment of phage was not seen with any of the PhD libraries, but the f884 library biopanning displayed an increase in recovery of phage at round three.

7.2.2 Analysis of PhD 10B2 Clones

Individual clones from the last round of PhD12 and PhDC7C biopanning on 10B2 were isolated and analysed via TRF and competition assays (figure 7.1). Of the nine clones isolated from the PhD12 library, five displayed higher binding to 10B2 than the isotype control and were specifically competed by Der p 2 for 10B2 binding.

Fifteen clones from the third round of biopanning with the PhDC7C library were also analysed. While these clones all exhibited specific binding to 10B2 in a TRF assay, when analysed in a competition assay, only seven were competed specifically, and not to the same degree as the PhD12 clones. All of the other clones were not competed, and therefore deemed to represent nonspecific immunoglobulin binding sequences and discarded.

Sequence analysis of the peptides displayed by the clones isolated from the PhD12 and PhDC7C libraries was performed (figure 7.2). The clones from the PhD12 library displayed a consensus sequence, DPN - - HYT -, a peptide similar to residues 69-82 of the Der p 2 primary sequence. The PhDC7C clones displayed a different consensus
sequence, though this peptide did not share any similarity with the Der p 2 primary sequence.

7.2.3 Analysis of f884 10B2 Clones

Ten clones from the third round of f884 biopanning against 10B2 were purified and analysed in TRF and competition assays. Results of these assays are shown in figure 7.3. All ten clones displayed specific binding to 10B2 in the TRF assay and a high degree of antigenicity. Aliquots of the phage pool after each round of biopanning were also analysed in TRF; increased binding was seen with each round, indicating enrichment for 10B2 specific clones. Nine of the ten clones were specifically competed by rDer p 2 for binding to 10B2.

Sequence analysis of the peptides displayed by the f884 clones revealed a consensus sequence shared by the nine specific clones, DP - - - HY (Figure 7.4). As with the PhD12 clones, this sequence displayed similarity with Der p 2 residues 69-82. The nonspecific clone 3.1 did not share any residues of this consensus sequence.

7.2.4 Immunisation of mice with 10B2 phage clones

Four clones were selected from the f884 and PhD12 10B2 clones for the investigation of immunogenicity. The phage were amplified in large scale culture and purified by density gradient centrifugation with cesium chloride before being used to immunise mice (figure 7.5). Only two clones induced Der p 2 specific antibodies, f884 3.5 and PhD12 3.1. In each case, high titres of anti-phage antibodies were generated in some mice but the antibody response was not consistent between individual mice. The immunisation was performed either with adjuvant via the subcutaneous route or without adjuvant via the intraperitoneal route. Phage clones isolated from the f884 library only induced specific antibodies when delivered by the intraperitoneal route, while PhD phage clones were immunogenic only when delivered via the subcutaneous route with adjuvant, although no firm conclusion can be reached because of the variation (see further work in section 7.2.5).
A ClustalW alignment of all of the peptides selected from the f884 and PhD12 libraries and part of the primary sequence of Der p 2 (including the residues 69-82) is shown in figure 7.6. A consensus sequence was observed between the peptides and Der p 2, DPN--HY. Examination of the two immunogenic clones revealed that they also shared this consensus sequence while the two peptides that did not induce antibody lacked the asparagine. The position of this region of Der p 2 was identified on the three-dimensional model (figure 7.7); most of the residues are surface exposed and located on a flexible loop, indicating potential accessibility for antibody binding.

### 7.2.5 Immunisation protocols

As the phage immunisation experiments above resulted in erratic antibody responses, it was decided to perform a series of experiments optimising immunisation protocols. The T7415 vector was selected for these experiments because it is a high copy vector, and therefore delivers a significantly higher amount of displayed peptide to animals.

Two T7 phage clones were selected for use in these experiments. The first expressed a peptide representing the residues 69-82 (DPNACHYMKCPLVK) of the Der p 2 primary sequence (T7P2c2) and the second clone, pan7, was selected from the T7 phylomer library in a previous biopanning experiment with the mAb 10B2 (T. Heinrich, personal communication). The pan7 clone expressed the peptide DPNSRHYLVAE, a sequence which shared the same consensus as clones selected from the f884 and PhD12 libraries. Pilot experiments had demonstrated that both of these phage clones were immunogenic.

The first variable investigated was the effect of phage dose and immunisation route on the antibody response, the results of which are shown in figure 7.8. The dose response experiments were carried out with T7P2c2, with phage delivered via the intraperitoneal route without adjuvant or via the subcutaneous route with adjuvant. At this initial phase only small groups (two to three) of C57BL6/J mice were used. The subcutaneous route induced only low titres of Der p 2 specific antibodies and only at the highest dose. Phage were injected via the intraperitoneal route elicited higher titres of
anti-Der p 2 antibodies, but only at the two highest doses of phage ($10^{10}$ and $10^{11}$ pfu). While these results were encouraging, the response of individual mice revealed a high degree of variability, as shown in the titration (figure 7.8B).

As the use of peptides displayed on the intact T7 phage resulted in inconsistent antibody responses, it was decided to perform a series of experiments using phage disrupted by treatment with SDS (figure 7.9). Groups of five mice from C57BL6/J, BALB/c and CBA strains were immunized with SDS treated phage (approximately $10^{11}$ pfu) expressing Der p 2 residues 69-82 or the Der p 2 mimotope pan7. All of the strains produced anti-Der p 2 antibodies in similar titres in response to both the P2 69-82 and pan7 immunisations. The titres tended to be higher than non-SDS treated phage immunised mice, but the difference was not statistically significant. All of the group of five mice immunised with SDS treated phage developed an anti-Der p 2 response, while only four of the group of five mice immunised with non-treated phage responded.

While SDS treatment of phage tended to increase the antibody titre, there was still variability in the responses within groups of mice. A series of 10B2 reactive sequences (Der p 2 69-82, plus the potential mimotope sequences f884 3.9, f884 3.8 and f884 3.2) were cloned into the pGEX plasmid and expressed as GST fusion proteins. Fifty micrograms of the GST fusions were used to immunise groups of five C57BL6/J mice via the subcutaneous route with Freund’s adjuvant; the results of this experiment are shown in figure 7.10. High titres of anti-Der p 2 antibodies were achieved when mice were immunised with the Der p 2 69-82 (GST P2) fusion, with far less variability between individual mice than was seen with the phage immunisations. In contrast to the phage immunisations, the GST P2 immunisation induced high anti-Der p 2 titres after only one dose. None of the GST-mimotope fusions resulted in a significant production of anti-Der p 2 antibodies.

### 7.2.6 Effect of epitope immunisation on sensitisation to allergens

In order to further elucidate the properties of the Der p 2 epitope 69-82, the effect of immunisation with a GST fusion of this peptide upon a murine model of allergic sensitisation was examined. Groups of C5BL6/J mice were immunized with size-
fractionated GST fusion proteins with complete Freund’s adjuvant via the subcutaneous route, boosted using incomplete Freund’s adjuvant. After a period of ten days the mice were sensitised via the intraperitoneal route with nDer p 2 or papain (as a specificity control) in alum adjuvant. The mice were bled at two and four weeks after the last boost, then challenged intranasally with three daily doses of 40μg of rDer p 2 (produced in *Pichia pastoris*) or papain to measure lung inflammatory responses. A bronchoalveolar lavage (BAL) was performed and serum IgE and IgG levels determined by PCA and TRF respectively.

The effect of the fusion protein on antibody production is shown in figure 7.11. After intraperitoneal sensitisation, all mice displayed high titres of IgG1 and IgG2a antibodies specific for the sensitising protein. In both cases, treatment with GST-P2 69-82 did not affect specific IgG1 or IgG2a production. Specific IgE levels were also unaffected by GST-P2 69-82 treatment.

While no significant differences were seen in antibody levels to the sensitising proteins, there were differences observed in the cell counts from BAL fluid in the Der p 2 group (figure 7.12). When the anti-Der p 2 response GST treated group was compared to the untreated control, significant increases were seen in the total cell number, eosinophils and neutrophils, indicating that the immunisation with the GST protein was acting to increase inflammation in the lungs. In the GST Der p 2 69-82 treated group, significant increases even above the GST controls were seen in total cell number, lymphocytes, eosinophils and neutrophils; the lymphocytes and eosinophils were significantly higher. No significant differences in cell numbers were observed in the adjuvant alone and papain sensitised groups.

### 7.2.7 Specificity of GST-Der p 2 69-82 immune serum

The specificity of GST-Der p 2 69-82 immune serum was determined via a competition assay. Serum was added to Der p 2 coated wells, with competitor added to duplicate wells (figure 7.13). 81% inhibition of antibody binding by Der p 2 was observed with GST Der p 2 69-82 immune serum, indicating that antibodies specific for Der p 2 had
been generated by immunization with this fusion protein. No significant inhibition was observed with the control protein, ovalbumin.

7.2.8 Competition of monoclonal antibodies DpX and 10B2

As the putative binding site of 10B2 identified in this study overlaps with the known binding site of the mAb DpX, a competition assay was performed with these two antibodies, the results of which are shown in figure 7.14. A fixed amount of one of the antibodies conjugated with biotin was competed with serial dilutions of the second antibody, with the biotin-labelled antibody detected by TRF. Interestingly, only a small amount of competition was seen, indicating that both of these antibodies are possibly able to bind to Der p 2 simultaneously.
7.3 DISCUSSION

This chapter describes the isolation of phage clones from random peptide phage display libraries using an anti-Der p 2 mAb as a biopanning target. This is the first application of random peptide phage display library screening to the identification of Der p 2 antibody epitopes and potential mimotopes.

Analysis of the phage clones selected from the f884 and PhD12 libraries revealed the presence of a consensus sequence that shared strong similarity with Der p 2 residues 69-82. A clone that were not specific for the Der p 2 mAb 10B2 did not share this consensus sequence. The fact that similar sequences were isolated from two different libraries also strengthens the relevance of this sequence. A similar sequence, pan7, had also been previously isolated from the T7 phylomer display library (T. Heinrich, personal communication).

Biopanning with the PhDC7C library resulted in the isolation of clones specific for 10B2 in an TRF assay, though only a small increase in phage percentage recovery over the rounds of biopanning was observed. However, when a competition assay was performed, only a few of the clones were competed by Der p 2, and only to a small degree. Sequence analysis of this clones revealed no similarity to the Der p 2 primary sequence, though a consensus sequence was present. It is possible that these clones display peptides reactive with the non-complementary binding regions of the mAb. Due to the lack of strong competition when compared to other more promising clones, these phage clones were not examined further.

Examination of the phage enrichment data over the rounds of biopanning on 10B2 revealed a similar pattern as to that which had been seen with FLAG M2 biopanning. Biopanning of the f884 library resulted in a significant enrichment of phage and the isolation of useful clones. In contrast, biopanning of the PhD7 and PhDC7C libraries did not result in a significant enrichment of phage or the isolation of useful clones. The enrichment seen in biopanning of the PhD12 library was not significant, though useful clones were isolated from this library.
Of interest was the clone f884 3.8, which shared the DP - - - HY sequence as well as two cysteine residues with the Der p 2 primary sequence. It has previously been reported that all of the disulphide bonds of Der p 2 are needed for antibody binding (Hakkaart et al., 1998.). The f884 3.8 clone did not display any significant difference to clones lacking the cysteines in vitro, indicating that it could bind the 10B2 mAb similarly to the other clones, and that these cysteines were possibly not required for antibody binding. It is also possible that the scaffold of the phage virion acted to force the other peptides into a rigid conformation in much the same manner as the disulphide bond. Examination of these peptides in other display vectors would clarify the role of these cysteine residues in antibody binding.

Residues 69-82 of Der p 2, which shared similarity to the phage clones, were identified on the three dimensional model of the protein. The peptide was located on a flexible loop region and was mainly surface exposed, and hence theoretically available for antibody binding. This sequence also represents part of the previously identified epitope of the anti-Der p 2 mAb DpX (Mueller et al., 2001). This region is conserved between Der p 2 isoforms, and is also mostly conserved between Der p 2 and Der f 2 (Smith et al., 2001), though the DPN motif is replaced by DTN in Der f 2. Indeed, the core mouse monoclonal antibody binding region of Der f 2 includes this region (Takai et al., 1997).

While immunisation with phage displayed vectors is a good primary screening method for the potential immunogenicity of the displayed peptides, the responses mounted by mice are erratic. It was therefore decided to undertake a series of experiments with phage-displayed peptides known to be immunogenic in order to investigate factors influencing immunisation success. The T7415 vector was chosen for these experiments because it is a high copy vector which replicates in vitro rapidly, facilitating the investigation of many different variables. The displayed peptides chosen included the Der p 2 69-82 peptide, and the Der p 2 pan7 mimotope, both of which had been previously identified as being immunogenic in display on the T7 vector. A pilot experiment appeared to show that higher doses of phage were more successful for immunisation with the intraperitoneal route. Subcutaneous immunisation did not show a dosage effect or high titres with the higher doses used. Based on these results, high
titres of phage (10^{10}-10^{11} pfu) delivered via the intraperitoneal route is the most effective regime for T7-based immunisation.

In an effort to produce consistent antibody responses, the T7 phage used to immunise were first treated with SDS to denature the phage into its component proteins. While the antibody responses were more consistent than the experiments with whole phage, there was still variation between the individual mice in a group. An increased titre of antibodies was generated when the phage were pretreated with SDS, when compared to untreated phage, possibly arising through the adjuvant effect of SDS itself (Clausen et al., 2000). As particulate antigens are very sensitive to antibody-mediated feedback (Heyman, 2003), it is also possible that SDS treatment dispersed the proteins subunits and thus reduced this feedback, improving the anti-peptide response.

Oligonucleotide cassettes representing several of the 10B2 selected peptides were cloned into the pGEX vector and produced as recombinant GST fusion proteins. When these proteins were used to immunise mice, the only one that resulted in the generation of anti-Der p 2 antibodies was the Der p 2 69-82 peptide. The mice immunised with this fusion produced a high titre of specific antibodies after only a single dose of the protein. All of the mice within a group responded to the protein, without the variation seen in phage immunization, and the antibody responses were one hundred times higher. Yip et al. (2000) compared filamentous phage vectors with GST fusions for mimotope immunisation. Of the filamentous vectors examined, pVIII display vectors resulted in the generation of a specific antibody response after fewer boosts than pIII display vectors. When the phage vectors and GST fusions were compared, the GST fusions were superior in terms of both the magnitude and kinetics of the antibody response. GST fusions appear to be far superior than phage display vectors for mimotope immunisation, and have the added advantage that they are monovalent, and therefore unable to cross-link cell-bound IgE, making them safer than multivalent phage vectors. Phage displayed peptides, however, remain ideal for in vitro screening.

None of the other peptides cloned as GST fusions resulted in a specific antibody response. As the peptides were selected with the antibody while in display on the phage scaffold, it is possible that the conformation of the peptide in terms of this scaffold was important for antibody binding and immunogenicity. Binding of the mAb 10B2 to the GST fusion proteins was however, readily demonstrated in vitro using a TRF assay.
The peptides to be cloned as GST fusions were chosen on the basis of the sequence data in order to give a variety of sequences; at this time no phage immunisation data was available, and hence the two immunogenic clones were not included. Based on the immunogenicity of the GST Der p 2 69-82 fusion, the peptides encoded by these clones need to be produced as GST fusions and their immunogenicity assessed.

In order to determine the relevance of the Der p 2 69-82 peptide, its effect on a previously established allergen sensitisation protocol was examined. Animals were pre-treated by immunization with the GST fusion before being sensitised to Der p 2 or papain and later challenged with the same protein. The pre-treatment with GST Der p 2 69-82 did not affect the levels of anti-Der p 2 (or papain) IgG1, IgG2a or IgE. However, when the animals were challenged with Der p 2 or papain and the cell numbers within their lungs determined, differences were seen. The GST Der p 2 69-82 fusion resulted in significant increases in total cell numbers, lymphocytes, eosinophils and neutrophils within the lung. The GST control resulted in increases in total cell numbers and eosinophils, indicating that this protein alone was increasing inflammation within the lungs. It is possible that the presence of Freund’s adjuvant also had an effect on cellular infiltration (Toth et al., 1989). When the GST control and the GST Der p 2 69-82 proteins were compared, it was clear that the injection of Der p 2 fusion resulted in more augmented inflammation than the GST protein itself. This effect was antigen specific, only affecting the Der p 2 sensitised group and not the papain sensitised group. While the peptide was not capable of altering the antibody response to Der p 2, it served to enhance the cellular response to Der p 2. The exact mechanism operating in this experiment is unknown, but it can be theorised that this peptide is pro-inflammatory, acting to recruit cells into the lungs. T-cell epitopes of the peptide are not critical, as the GST protein itself contains T-cell epitopes (Li et al., 2004).

The residues 69-82 identified as part of the putative epitope for the mAb 10B2 overlap with the known epitope of the anti-papain mAb DpX. The DpX epitope includes the residues 47-52, 68-81, 84-91 and 110-112 (Mueller et al., 2001). The lack of blocking activity observed for the 69-82 peptide may reflect the involvement of residues distant from this peptide in binding of 10B2 to DpX. It has also been observed that human IgE directed against Der p 2 binds to residues 74-87 (Joost van Neervan et al., 1993) and 65-78 of Der p 2; this interaction was readily inhibited by nDer p 2 (van't Hof et al., 1991).
It is possible that the addition of residues 65-68 to the Der p 2 peptide used in these experiments would increase the chance of raising antibodies capable of completely blocking the human IgE-Der p 2 interaction. It would also be of interest to examine the affinity of the peptide 69-82 for antibody binding, and compare it to overlapping peptides representing this whole region of Der p 2. It is probable that the effect of this peptide is an epitope specific effect, and that other epitopes of Der p 2 exist which would be capable of inducing blocking antibodies.

Of interest was the fact that the 10B2 and DpX antibodies do not strongly compete each other for binding to Der p 2. Mueller et al. (2001) previously demonstrated that the antibodies DpX and 6D6 were able to bind to Der p 2 simultaneously, though their epitopes overlap. DpX binds to Der p 2 residues 47-52, 68-81, 84-91 and 110-112, while 6D6 binds to residues 7-13, 41-55, 70-73, 76, 80 and 107-120. It is possible that a similar situation exists with DpX and 10B2, where their epitopes overlap, but enough core binding residues are still available for the binding of both antibodies.
Table 7.1 Enrichment of phage over 10B2 biopanning.

Figures shown are the percentage recovery of phage, calculated by dividing the output titre by the input titre and multiplying by 100. A significant increase is defined as a greater than hundredfold increase between rounds.
Figure 7.1 Analysis of PhD12 and PhDC7C 10B2 phage clones.
Panel A shows a TRF analysis of PhD12 and PhDC7C clones from the third round of biopanning against the mAb 10B2 and an isotype control mAb. Panel B shows a competition assay of the same clones. The amount of rDer p 2 added per well was 0, 250, 500 or 1000ng.
**Figure 7.2 Alignment of PhD12 and PhDC7C clones with Der p 2**

The sequence of the displayed peptide for each of the clones is shown. A ClustalW alignment with part of the native Der p 2 sequence, P2 (residues 58-89) is shown. Identities or unmatched are highlighted in dark grey, and similarities in light grey. Panel A shows the PhD12 clones, panel B PhDC7C clones. The consensus sequence of the alignment is shown at the bottom of the alignment.
Figure 7.3  Analysis of f884 10B2 phage clones

Panel A shows a TRF of third round clones as well as total eluates from the rounds of biopanning and an aliquot of unselected library (f884) against the mAb 10B2 and an isotype control antibody. Panel B shows a competition TRF of the ten third round clones, where 0, 0.5, 1 or 2μg of rDer p 2 was added per well.
Figure 7.4 Alignment of f884 clones and Der p 2.

The displayed peptides are shown in a ClustalW alignment against part of the native Der p 2 sequence (residues 58-89). Identities, similarities or unmatched are highlighted in grey. The consensus sequence is shown at the bottom of the alignment. Panel A shows the alignment of the nine specific clones, while panel B shows the alignment of the non-specific clone 3.1.
Figure 7.5 Immunisation with PhD and f884 10B2 clones.

Groups of three mice were immunised with approximately $10^{11}$ pfu of phage via the intraperitoneal (ip) or subcutaneous (sc) route. Mean titre is shown with standard error. Statistical significance could not be determined because of the size of the animal groups. M13k07 is a control filamentous phage displaying no peptide.
**Figure 7.6 Alignment of PhD and f884 10B2 clones with Der p 2.**

The displayed peptide sequences are shown in a ClustalW alignment with residues 58-89 of Der p 2. Identities, similarities or unmatched are highlighted in grey. f884 phage clones are labelled “translation of”. Immunoogenic clones were PhD12 3.1 and f884 3.5, marked with a star. The PhDC7C clones are not shown, as they do not align with the Der p 2 primary sequence. The consensus sequence is shown beneath the alignment.
Figure 7.7 10B2 Peptide on the 3D Model of Der p 2.

The three-dimensional model of the Der p 2 protein is shown (PDB accession number 9PAP). Panel A shows the protein backbone in spacefilling mode, with residues 69-82 in spacefilling mode and highlighted in yellow. Panel B shows the protein backbone as a ribbon, with residues 69-82 in spacefilling mode.
Figure 7.8  T7 immunisation: Dose response and route of delivery.

Panel A shows mean IgG titres against Der p 2 with standard error. Statistical significance were not determined because there were only two to three animals per group. Panel B shows the titration curves of individual mouse serum from the intraperitoneal $10^9$pfu and $10^{11}$pfu groups.
Figure 7.9  T7 immunisation: Strain effects.

Groups of five mice were immunised with approximately $10^{11}$ pfu of phage treated with SDS, emulsified in adjuvant and administered via the subcutaneous route. Mean titres are shown with standard error. The “No SDS” group received the P2 69-82 T7 clone without SDS pre-treatment.
Figure 7.10  GST mimotope immunisation.

Groups of three mice were immunised with GST fusion proteins emulsified in adjuvant and delivered via the subcutaneous route. Mean IgG titre against Der p 2 is shown with standard error (Panel A). Panel B shows individual titration curves against Der p 2.
Figure 7.11  GST P2 69-82 effect on allergen sensitisation.

Serum was assessed for IgG1 and IgG2a binding to the sensitising allergen (Der p 2 or papain). Mean titres are shown with standard error. B1 represents bleed one and B2 bleed two (at two and four weeks after sensitisation respectively. The GSTP2pep group were pre-treated with the GST fusion with P2 69-82. The control group received no GST fusion pre-treatment, while the GST group received the unfused GST protein.
Figure 7.12 GST P2 69-82 effect on sensitisation to allergens.

Groups of mice were immunised with GST fused to P2 residues 69-82 (GST-2) or unfused GST or left untouched before sensitisation. Average cell number are shown, with standard error; significant differences are indicated by a star. Panel A shows the results of challenge with Der p 2, panel B the results of challenge with papain.
Figure 7.13  Competition of GST-Der p 2 69-82 serum by Der p 2.

A 1:100 dilution of mouse serum was added to Der p 2 coated wells. Twenty-three micrograms of competitor (Der p 2 or ovalbumin) was added. GST P2c2 = anti-GST Der p 2 69-82 immune serum, GST = anti-GST immune serum.
Figure 7.14 Competition of mAbs 10B2 and DpX for Der p 2 binding.
A constant amount of the biotinylated antibody was competed with dilutions of the competing, non-biotinylated antibody. Bound biotinylated antibody was detected in the TRF. None = no competitor added.
CHAPTER EIGHT

GENERAL DISCUSSION
8.1 DISCUSSION

The only method that has the potential to act as a causative treatment of allergic disease, including allergic asthma, is allergen immunotherapy. Conventional SIT typically involves the repeated subcutaneous administration of increasing doses of allergen extracts. While this methodology has demonstrated some success, the use of allergen extracts carries the risk of anaphylaxis, as well as the potential for the development of new allergies to other allergens present within the extract (Lewis, 2002). The availability of recombinant allergens has facilitated the use of allergens in immunotherapy at higher purity to reduce the risk of developing novel allergies, but the risk of anaphylaxis still exists. There is therefore a need for the development of safer reagents for use in allergen immunotherapy. Mimotopes, peptides that mimic allergen epitopes, are one such potential reagent.

The screening of random peptide phage display libraries using antibodies as a target has become one of the preferred methods for the identification of mimotopes as well as the mapping of antibody epitopes. Several different methodologies have been used to screen phage display libraries, including the use of both polyclonal human serum (Folgori et al., 1994; Gevorkian et al., 1998) and monoclonal antibodies directed against the antigen of interest. The choice of monoclonal antibody to be used as a biopanning target can be based on biological properties, for example their ability to neutralise virus (Yu et al., 2000), or their ability to inhibit human antibody binding (McElveen et al., 1998; Furmonaviciene et al., 1999).

To date, the application of phage display libraries and mimotopes to the study of allergens has been limited. The screening of phage display libraries has facilitated the identification of a putative human IgE epitope on Der p 1 (Furmonoviciene et al., 1999), the isolation of a plant profilin mimotope able to inhibit the binding of human IgE (Leitner et al., 1999), the identification of a grass pollen allergen mimotope able to inhibit the binding of human IgE (Suphioglu et al., 2001), the mapping of conformational IgE epitopes of the grass pollen allergen Phl p 5 (Hantusch et al., 2004).
and the identification of Bet \( \text{v} \) 1 mimotopes using human IgE (Ganglberger et al., 2000) or murine monoclonal antibodies (Jensen-Jarolim et al., 1998) as a biopanning target.

The major drawback to most of the above studies is that the identified mimotopes were only examined \textit{in vitro}; immunogenicity was not examined for the Der \( p \) 1, profilin or grass pollen mimotopes. The only allergens for which an in depth study of mimotopes, including immunogenicity, has been made are Bet \( \text{v} \) 1 and Phl \( p \) 5a. IgE-selected Bet \( \text{v} \) 1 mimotopes have a demonstrated ability to induce the production of blocking antibodies (Ganglberger et al., 2000) and have also successfully been utilised for immunisation in monovalent display on an albumin binding protein (Ganglberger et al., 2001). Mouse monoclonal antibody-selected Bet \( \text{v} \) 1 mimotopes have been demonstrated to be immunogenic and able to directly inhibit the binding of anti-Bet \( \text{v} \) 1 mAbs and Bet \( \text{v} \) 1 (Jensen-Jarolim et al., 1998). Mimotopes of Phl \( p \) 5a have been demonstrated to be immunogenic, and to be capable of inhibiting the binding of anti-Phl \( p \) 5a IgE to Phl \( p \) 5a (Hantusch et al., 2004).

While the Bet \( \text{v} \) 1 and Phl \( p \) 5a studies provided encouraging data for the potential development of biologically active mimotopes, and the other allergen mimotope studies demonstrated that it was possible to identify mimotopes with \textit{in vitro} activity, the lack of \textit{in vivo} data for the latter group casts doubt on their use as either clinical or diagnostic reagents. Further, the protocols utilised in these and other mimotope studies have varied widely, with no real consistency or analysis of the factors involved in the successful isolation of mimotopes.

Successful allergen immunotherapy is associated with the development of IgG antibodies capable of inhibiting the allergic response at the cellular level, though they do not have an effect on IgE levels. Immunisation with allergen mimotopes is envisaged to act at least in part by the development of blocking antibodies. In order to investigate potential blocking antibodies \textit{in vivo}, four different anti-papain monoclonal antibodies were produced and administered to mice either before allergen sensitisation or allergen challenge. Three of the antibodies examined possessed the ability to inhibit inflammation within the lung, though the profile of inhibition varied for each of the antibodies, presumably reflecting differing epitope specificities. These results are extremely important, demonstrating that antibodies are capable of inhibiting allergic inflammation \textit{in vivo}. The antibodies were capable of inhibiting allergen sensitisation,
indicating that blocking antibodies could be generated by prophylactic vaccination, potentially using mimotopes. A subset of the antibodies were also capable of inhibiting inflammation in a previously sensitised animal, indicating that blocking antibodies generated during immunotherapy could act to decrease the allergic response.

One such strategy to be used in the thesis was to utilise the anti-papain antibodies possessing *in vivo* activity for the study of mimotopes. Unfortunately, it was the non-blocking anti-papain antibody for which phage clones were isolated, biopanning with the other three antibodies resulting only in the isolation of non-specific immunoglobulin-binding clones. Furthermore, the mimotopes isolated with the non-blocking mAb were non-immunogenic. The lack of immunogenicity could now be revisited to apply the experience obtained with later models but this was not possible with the time available for this study. Further biopanning experiments with other phage display libraries and the three biologically active (in terms of blocking ability) antibodies would also be desirable and perhaps it is necessary to use larger amounts of antibody. Papain mimotopes isolated using these antibodies could be examined in the context of the existing papain model of allergic sensitisation.

Analysis of some of the parameters that define a successful biopanning reaction were made utilising the FLAG epitope as a model. The well-defined nature of this epitope, as well as its high affinity of monoclonal antibody binding and commercial availability, made it a good choice for this analysis. A significant increase in phage recovery was only observed when clones with the FLAG sequence (or a variant of the FLAG sequence) had been isolated, confirming that an increase in the percentage of phage recovery is associated with the enrichment of the phage pool for specific clones. The importance of screening individual clones in both a TRF assay against the target antibody and an isotype control antibody and a competition assay was demonstrated, with isolated phage clones displaying a variety of binding properties.

The FLAG biopanning experiments also demonstrated the importance of screening multiple libraries. Screening of the PhD12 library did not result in the isolation of specific clones, while the PhD7 library yielded clones of low antigenicity and no immunogenicity. The f884 library, in direct contrast, resulted in the isolation of clones of high antigenicity. Of the two clones screened for potential immunogenicity, one was able to induce antibodies capable of cross-reacting to the canonical FLAG peptide.
The presence of a consensus sequence between isolated phage clones and the original antigen is often used as a measure of the success of a biopanning experiment. The tendency of the phage clones to display a similar primary sequence to the original antigen has been termed “convergent evolution” by Kay et al. (2000). Similarity between mimotopes and the original antigen has been observed for example, with hepatitis B surface antigen (Meola et al., 1995), and glycoprotein G from herpes simplex virus (Grabowska et al., 2000). Other studies have isolated clones that do not display similarity to the primary sequence of the original antigen. For the allergens Der p 1 (Furmonaviciene et al., 1999) and Bet v 1 (Ganglberger et al., 2001), no similarity was observed between the mimotope sequences and the primary sequence of the original antigen. In each case, the authors used the mimotope sequences to identify a putative conformational epitope on the three-dimensional model of the allergen.

Successful biopanning with the FLAG M2 monoclonal antibody and f884 library resulted in the isolation of clones that demonstrated similarity to the primary sequence of the canonical FLAG sequence. A previous study had also observed this similarity, and had gone on to further define the residues critical for antibody binding (Miceli et al., 1994). These residues were identical to those in the consensus sequence of the f884 FLAG clones.

While there was a clear trend for the association of the isolation of antigenic clones with a significant increase in the percentage of phage recovery over the rounds of biopanning, there was no correlation between the antigenicity and immunogenicity of a clone. The two FLAG variant f884 clones examined for immunogenicity, for example, shared similar antigenicity, but only one of these clones was immunogenic. Previous studies have also noted the lack of correlation between antigenicity and immunogenicity. Grabowska et al. (2000) isolated mimotopes of glycoprotein G from herpes simplex virus; the most antigenic clone in an ELISA was in fact the least immunogenic. It has also been observed that phage clones that do not demonstrate any antigenicity in vitro can actually be immunogenic and capable of raising antibodies cross-reactive against the original antigen (El Kasmi et al., 1999). This data, along with the data from the FLAG biopanning experiments, emphasise the importance of screening a panel of phage clones for both antigenicity and immunogenicity.
The results generated from the FLAG biopanning experiments allowed a further examination of the equivocal results obtained from biopanning using anti-papain monoclonal antibodies as targets. No significant increases in phage recovery were observed for any of the libraries utilised, and the clones isolated from the final rounds of biopanning demonstrated very low antigenicity. Together, these two factors indicate that the biopanning reactions had been unsuccessful in the isolation of papain mimotopes; indeed, the two clones examined further did not demonstrate immunogenicity.

While the FLAG biopanning allowed for an examination of the biopanning process, the use of the phage clones beyond this point was limited, although the possibility of using FLAG-tagged recombinant allergens for \textit{in vivo} experiments was contemplated. Biopanning for mimotopes of the house dust mite allergen Der p 2 was, however, successful, so this possible strategy was obviated. Four different phage display libraries were biopanned, only two of which (f884 and PhD12) resulted in the isolation of potentially useful clones. Specific phage clones isolated from these two libraries were highly antigenic. Examination of the displayed peptides revealed a strong consensus sequence that was shared between the two libraries; this consensus displayed similarity to the Der p 2 primary sequence from residues 69-82. This region has been previously been identified as being part of a murine monoclonal antibody epitope (Derewenda et al., 2002). Binding of this region to the mAb was very high, similar to the binding of the entire Der p 2 protein to the mAb.

Two of the Der p 2 peptide phage clones demonstrated immunogenicity in a mouse model, inducing the production of antibodies cross-reactive to Der p 2. The differences in immunogenicity between the clones tested could have been due to variations in affinity or the effect of flanking residues. The affinity of a mimotope has previously been associated with the degree of protection inferred against viral infection conferred by mimotope immunisation (Olszewka et al., 2000). Flanking residues also have a marked effect on immunogenicity (Vijayakrishnan et al., 1997) and on the affinity of the raised antibodies (Ferrieres et al., 2000).

A series of experiments utilising the Der p 2 69-82 peptide and Der p 2 pan7 mimotope in display on T7-415 phage were carried out. Several general trends were observed, including the generation of higher antibody titres with a higher dose of phage, but a
high degree of variation between individual animal response was observed. More consistent results were achieved when the phage were treated with SDS before administration to animals, and the data suggested that the procedure should be followed up for general application. The processing of T7 phage has been studied in rats (Sokoloff et al., 2000); it can be assumed that a similar process takes place in mice. The half life of T7 in the rat serum was less than three minutes, with phage inactivation mediated by complement and naturally occurring antibodies. The increase in consistency seen with the use of SDS-disrupted phage may have occurred due to slower processing of phage or a reduction in antibody-mediated feedback or a dispersal of phage coat proteins. Particulate antigens are very sensitive to the last (Heyman et al., 2000).

Immunisation with the filamentous phage vectors generated similar results, with a significant degree of variation between individual animals making the determination of statistical significance impossible. A possible trend of pVIII display immunisation resulting in higher antibody titres than pIII display immunisation was seen. This has been observed previously by Greenwood et al. (1991) and attributed to the higher copy number of displayed peptides on the pVIII vectors. The variation in individual animal responses to pVIII display vectors could be explained in part by the fact that the expression level of the recombinant peptide can vary markedly from virion to virion, with anywhere from ten to thirty percent of the total surface protein made by of the recombinant peptide (Molenaar et al., 2002).

The position of residues 69-82 on the three-dimensional structure of Der p 2 showed that the putative epitope was located on a flexible surface-exposed loop, theoretically accessible for antibody binding. It incorporates the only turn in the entire molecule. This region overlaps with previously defined epitope of another anti-Der p 2 mAb, DpX. The DpX epitope contains the linear region 69-82 as well as other conformational components (Mueller et al., 2001). The lack of strong competition between DpX and 10B2 for Der p 2 binding indicates that other regions outside of the 69-82 residues are involved in the 10B2 epitope. The strong antibody binding of the 10B2 mAb to peptides lacking the two cysteine residues within 69-82 is of interest, showing that the loop for residues 73-78 is not required for the epitope.
While immunisation with phage-displayed mimotopes is attractive for an initial screen for immunogenicity, it was not optimal for several reasons. First, the variation in immune responses observed for both T7 and filamentous phage make it difficult to determine statistical significance. Second, the use of a monovalent display vector is critical for eventual clinical use of a mimotope, as multivalent display can result in cross-linking of mast cell-bound IgE, leading to degranulation of cells and, eventually, anaphylaxis. It may be preferable to examine the immunogenicity of monovalent constructs at an early stage. The GST fusion partner was selected for use, as it had previously been used for successful immunisation with gene fragments of an epidermal growth factor receptor (Yip et al., 2001).

The GST fusion of Der p 2 69-82 resulted in an extremely high titre of anti-Der p 2 antibodies after only a single dose. This result was replicable and the response was sustained and comparable between individual animals, allowing the determination of statistical significance. Immunisation with the GST fusion was therefore deemed to be superior to any of the other protocols investigated, although clearly this needs to be tested with peptides. Successful immunisation with a peptide in monovalent display is an extremely important step in the development of mimotopes as vaccines. There have only been a few studies that have successfully immunised with mimotopes/epitopes in monovalent display. Ganglberger et al. (2001) successfully transferred a Bet v 1 IgE mimotope from a phage display vector to display on an albumin binding protein. This fusion protein was immunogenic and deemed safe for use in an animal model, producing no anaphylaxis or other unwanted side effects. A fragment of the protein ErbB-2 has also been used to successfully immunise mice in monovalent display on the GST protein (Yip et al., 2001).

Further investigation into the Der p 2 69-82 GST fusion was made by examining its effect on a murine model of allergic sensitisation. To date, no other studies have investigated the effect of a mimotope or epitope on the process of allergic sensitisation in an animal model. If an epitope or mimotope was protective, one would expect to see a decrease in allergic sensitisation in animals pre-treated with the epitope. In the case of the Der p 2 69-82 peptide, the opposite occurred. While there was no effect on the antibody response, an increase in cellular infiltration into the lung occurred. Specifically, the GST Der p 2 69-82 fusion resulted in increases in the lymphocyte and eosinophil numbers in the lungs, indicating an increase in both inflammation and
allergic sensitisation. The lack of effect on the IgE levels was not completely unexpected, as even successful immunotherapy does not usually result in overall changes to serum IgE levels (Weber, 1997).

Antibodies directed against Bet v 1 have been identified that are capable of both blocking the binding of IgE to the protein or enhancing IgE binding (Jarolim et al., 1989). Work in this thesis with anti-papain mAbs has also demonstrated that antibodies directed against the same target can possess a range of biological activities. It follows therefore that antibodies directed against Der p 2 would also possess a range of activities, and would therefore include both blocking and enhancing antibodies. The Der p 2 mimotope work presented in this thesis involved a single antibody only. Other antibodies with different epitope specificities could be expected to generate different results.

Murine IgG antibodies have the potential to be both anaphylactic and nonanaphylactic, and the potential of peptides to raise either class of antibody is reliant on the peptide sequence. As the GST Der p 2 69-82 fusion did not result in any changes to the titre of IgG1, IgG2a or IgE produced, it is possible that it instead resulted in a change in the properties of the antibodies generated, preferentially inducing the production of anaphylactic antibodies. It has been demonstrated previously that murine chimaeric antibodies of both IgG1 and IgG4 subclasses that bind to Der p 2 IgE epitopes are capable of increasing the activation of sensitised basophils (Schuurman et al., 1998). It is also known that immune complexes can stimulate the synthesis of prostaglandin E2 and the release of IL-6 and IL-10 in a dose and antigen:antibody ratio-dependent fashion (Berger et al., 1996), as well as decreasing the production of IL-12 (Anderson et al., 2004). The action of the fusion protein was specific to Der p 2; it is known that enhancement by antibody is antigen specific and mediated by FcγRs on macrophages, monocytes and neutrophils (Ravetch and Bollard, 2001). In particular, anaphylactic IgG1 binds FcγRIII on mast cells, activating them (Macedo-Soares et al., 2001).

The region 69-82 of Der p 2 is part of a binding site for both murine IgG and human IgE, and also represents a T-cell epitope in some individuals (Joost van Neervan et al., 1993). The strong selection of mimotopes of this region, immunogenicity of the peptide fusion, and enhancement of allergic sensitisation produced by pre-treatment with the peptide fusion all indicate that this epitope is of critical importance in the development
of allergy to Der p 2. The lack of strain differences observed in mice when immunised with the peptide in display on SDS-treated T7 phage, indicates that the antibody response to this peptide is not dependent on the specific VH genes, and that this peptide may represent a universal epitope.

This study has demonstrated the ability of antibodies directed against a single epitope to inhibit allergic inflammation when administered before and after sensitisation. It also showed that only some antibodies were inhibitory. The process of biopanning has been analysed by the use of a model epitope, and the factors involved in the successful isolation of specific clones identified. Phage clones isolated by biopanning with an anti-Der p 2 antibody have allowed the identification of a putative epitope (or part thereof) which is capable of inducing anti-Der p 2 antibodies. This epitope was also successfully used to immunise against Der p 2 in monovalent display. The immunised mice did not show reduced allergic responses, which along with the anti-papain monoclonal antibody studies, indicates the need to examine further Der p 2 epitopes.
CHAPTER NINE

REFERENCES


APPENDIX 1

DER P 1 MIMOTOPES
i INTRODUCTION

Der p 1 is a good candidate for the isolation of mimotopes for several reasons. The cDNA encoding the protein has been fully sequenced (Chua et al., 1988) and a three-dimensional model created by comparison with known crystal structures of related cysteine proteases (Topham et al., 1994). The protein itself is a major allergen of the house dust mite, and human Der p 1 allergic serum is readily available. The major issue with the use of Der p 1 in an experimental setting has been the difficulty in the preparation of a recombinant form. Mimotopes could potentially provide alternative reagents for Der p 1.

Antibody binding structures of Der p 1 have been investigated by several groups. Greene at al (1991) identified at least five different regions of the Der p 1 primary sequence which bound human IgE and IgG, comprising residues 1-56, 53-99, 98-140, 166-194 and 188-122. Further work by Greene and Thomas (1992) further defined IgE binding regions as residues 15-33, 60-80, 81-94, 101-111 and 155-187, all of which were located on the protein surface. Of particular interest were the three regions 60-80, 81-94 and 101-111, which were all brought into close proximity by protein folding. This region also bound strongly to IgG from hyperimmune rabbit serum (Greene et al., 1990).

The epitopes of Der p 1 recognised by human IgE all appear to be highly dependent on intact tertiary structure (Collins et al., 1996; Lombardero et al., 1990). Despite this, peptides representing sections of the Der p 1 primary sequence can show some serological reactivity. Peptides representing 52-71, 117-133, 176-187 and 188-199 are all capable inducing histamine release from basophils, indicating that these peptides represent parts of human IgE epitopes on Der p 1 (Jeannin et al., 1992).

As the human reaction to allergens is polyclonal, the use of human serum may not be the simplest system for the isolation of mimotopes. Instead, a mouse monoclonal antibody, 4C1, was used. This mAb was originally produced by Heymann et al (1986) by immunising BALB/c mice with Der f 1, and is cross-reactive between Der p 1 and Der f 1. Mouse monoclonal antibodies can readily inhibit IgE binding to Der p 1 (Lind et al., 1988), therefore the substitution of a murine mAb for human IgE is valid (Schou, 1995).
Only one previous study has used phage display libraries to investigate antigenic determinants of Der p 1. Furmonaviciene et al. (1999) used the murine mAb 2C7, previously demonstrated to inhibit human IgE binding to Der p 1 (McElveen et al., 1998), to screen phage display libraries. The antibody was mapped to the residues 147-160 of Der p 1, postulated to represent part of a conformational epitope. The authors also theorised that this epitope was cross-reactive between cysteine proteases from different species because similar sequences were identified in the three-dimensional models of chymopapain, papain and actinidin. Whether the peptide represents an immunogenic mimotope is unknown, because its ability to induce anti-Der p 1 antibodies was not investigated.

This appendix describes the use of random phage display libraries and the murine monoclonal antibody 4C1 to isolate peptide mimics of Der p 1, and to investigate the possibility that these peptides represent mimotopes.
### RESULTS

**PhD Library Biopanning**

The three different PhD phage display libraries were combined to give an initial input titre of $10^{11}$ pfu into the first round of biopanning. A significant increase in phage recovery was observed at round three (figure i).

**ELISA Screening of Clones**

Individual phage clones from the third round of biopanning were isolated and amplified on a small scale before being screened in an ELISA (figure ii). Of the fifty clones analysed, ten displayed higher binding to 4C1 than the isotype control and blocked plastic. This screening was repeated with a second batch of clones (results now shown) and six more clones exhibiting binding to 4C1 isolated.

**Competition ELISA**

The sixteen clones were analysed in a competition assay with Der p 1 and an ovalbumin control. The results of one clone, 4C1PhD1, are shown in figure iii. While the addition of Der p 1 resulted in decreased binding of the phage to 4C1, the competition did not titrate as would be expected, possibly indicating low affinity.

**Sequencing**

A selection of 4C1 selected PhD clones was sequenced using the –96 sequencing primer (figure iv). Out of seventeen clones, twelve of them expressed identical peptides (4C1PhD1), which originated from the PhD12 library. Two additional sequences were also from the PhD12 library, while the final two sequences were from the PhDC7C
library. There was no strong consensus sequence between the clones or between any individual clones and the primary sequence of Der p 1.

Immunisation

The phage clone 4C1PhD1 was selected for use in a pilot immunisation experiment because it was selected twelve times. Groups of three C57BL6/J mice were immunised with $10^{11}$pfu 4C1PhD1 with CFA via the subcutaneous route, and boosted at monthly intervals, substituting IFA for CFA. A further group of mice were immunised in a similar fashion with an unrelated control phage. Both groups of mice demonstrated only a low signal to Der p 1 (figure v), with no significant difference between them.

f884 Library Biopanning

The f884 phage display library was biopanned against 4C1 using an initial input titre into round one of $3.45 \times 10^7$pfu. The enrichment of phage over rounds of biopanning is shown graphically in figure 4.7; significant enrichment was seen at round three.

ELISA Screening of Clones

Individual clones from the third round of biopanning were isolated and amplified on a small scale before being screened in an ELISA. A total of eight clones were screened for binding to, an isotype control antibody, and blocked plastic (figure vii). Overall reactivity was low, though five clones exhibited higher binding to 4C1 than to the isotype control or blocked plastic.

Sequencing

The five specific phage clones isolated from the third round of biopanning were sequenced with the f884 sequencing primer. A ClustalW alignment of the displayed
peptides is shown in figure viii. A consensus sequence, - H – P – G – PT - - - - ILL, was present between all of the clones. The nonspecific clones were also sequenced, and did not share this consensus (data not shown).

ii DISCUSSION

Several different regions on the surface of Der p 1 have previously been identified as being capable of binding human, rabbit and mouse antibodies. Immunogenicity of some of these regions has been demonstrated in a rabbit model. These epitopes are all conformational, however the ability of several peptides from the primary sequence of Der p 1 to induce histamine release from basophils indicates that at least part of the epitopes are linear and possess biological activity (Jeannin et al., 1992).

The work of Furmonaviciene et al. (1999) demonstrated the possibility of using a murine mAb to biopan random phage display libraries to identify peptide mimics of Der p 1. The peptides isolated displayed a consensus sequence that aligned, using a “structural” algorithm, with the residues Val140-Tyr169 of Der p 1. These residues were located on a loop on the three-dimensional model of Der p 1, indicating potential accessibility to antibodies.

Biopanning with the anti-Der p 1 mAb 4C1 was undertaken using a series of filamentous phage display libraries. The fact that 4C1 cross-reacts between Der p 1 and Der f 1 facilitates the possibility that mimotopes could be selected that cross-react between both Der p 1 and Der f 1, though this was not examined in the scope of this thesis.

The PhD phage display libraries were biopanned in combination on the 4C1 mAb. Initial results looked promising, with a significant increase in phage enrichment over rounds of biopanning, and the identification of clones specific for 4C1 in an ELISA. Sequencing of the phage clones revealed six different peptide sequences, one of which was isolated from twelve individual clones. Competition of this clone with Der p 1 for 4C1 binding showed some weak inhibition, indicating that the displayed peptide may possess weak affinity for the paratope of 4C1. The clone 4C1PhD1 was selected for pilot immunization experiments because of its multiple isolation, however it did not
result in the generation of specific anti-Der p 1 antibodies. Either the phage-displayed peptide is not immunogenic, as has been observed with mimotopes of the pertussis toxin (Felici et al., 1993), or the immunization protocol was not optimal. Further experiments would confirm this.

Previous work by Greene (unpublished data) mapped the epitope of 4C1 on Der p 1 by the use of overlapping peptides representing the primary sequence of Der p 1. The antibody demonstrated specific binding for the region 60-80, indicating that at least part of the epitope was contained in this linear region. The 4C1PhD1 sequence did not display any direct similarity with this region. It is possible that the peptide is mimicking the shape of the epitope, or that it is mimicking other residues involved in the binding of 4C1 not directly identifiable from the primary sequence.

Biopanning on 4C1 was also performed using the f884 phage display library. Initial results were encouraging, with significant enrichment of phage seen at round three, and specific clones identified in an ELISA, though they displayed only slightly higher binding to 4C1 than to the isotype control, and overall reactivity was low. Sequencing of the specific clones revealed a consensus sequence that was not shared with the non-specific clones. However, when a competition ELISA was performed with these clones (results not shown), no specific inhibition was observed. Immunisation experiments were not carried out with these phage clones because of the lack of specificity.

It was therefore concluded that the clones isolated from the f884 library probably represented nonspecific immunoglobulin or plastic binding phage. Selection of plastic binding phage during biopanning has previously been observed (Adey et al., 1995), though the consensus sequences identified by these authors did not share similarity with the f884 peptides. It is probable that the f884 library does not contain any sequences capable of binding to 4C1 with high affinity.

Biopanning with the PhD and f884 phage display libraries failed to isolate any immunogenic mimotopes of Der p 1. This possibly occurred due to a lack of correct binding peptides within these libraries, or a rarity of specific peptides that were subsequently lost during the biopanning process. One Der p 1 mimotope was isolated from the PhD12 library, but appeared to be of low affinity and was not immunogenic. It
would be advantageous to screen other libraries with 4C1, or to utilise other mAbs
directed against Der p 1 for use in biopanning.
Figure i Enrichment of PhD phage over rounds of 4C1 biopanning.

The combined PhD libraries were biopanned against the anti-Der p 1 mAb 4C1. The percentage recovery of phage was determined by dividing the output phage titre by the input phage titre. A significant increase was defined as a greater than hundredfold increase.
Figure ii ELISA screen of individual clones from PhD 4C1 biopanning.

Individual clones were tested against 4C1, the isotype control antibody 7A1 or blocked plastic. Positive clones exhibited higher binding to 4C1 than 7A1 or blocked plastic.
**Figure iii Competition ELISA of PhD 4C1mim.**

nDer p 1 or ovalbumin (Ova) was added at 100ng/ml (dilution 1), with each successive dilution two-fold less than the previous. Dilution 0 represents the binding of the phage without competitor. Reactivity is to the mAb 4C1.
**Figure iv** ClustalW alignment of PhD 4C1mim clones.

The peptides displayed by the 4C1 selected PhD phage are shown in a ClustalW alignment. Identities, unmatched and similarities are shown highlighted in grey. 4C1 PhD1 was selected twelve times.
Figure v  ELISA of phage immune serum

Serum pooled from groups of three mice was assessed for binding to nDer p 1. The groups of mice were immunised with the 4C1 derived phage (4C1 phage) or an unrelated control phage. The initial serum dilution was 1/10 (point 1), with each successful point a further ten-fold dilution.
Figure vi  Enrichment of f884 phage over 4C1 biopanning.

The percentage recovery of phage was determined by dividing the output phage titre by the input phage titre.
Figure vii  ELISA of f884 4C1 biopanning clones

Individual phage clones were assessed for binding to 4C1, and isotype control and blocked plastic.
Figure viii ClustalW alignment of f884 4C1mim clones.

The peptides displayed by the 4C1 selected f884 phage are shown in a ClustalW alignment. Identities and similarities are shown highlighted in grey.