

The Application of Phage Display in Allergy Research: Characterization of IgE, Identification of Allergens and Development of Novel Therapeutics

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Abstract: The ability to display IgE antibody fragments, allergens and peptides upon filamentous phage has increasingly been used in allergy research. This technique offers the opportunity to isolate and produce IgE antibody fragments specific for allergens. These antibody fragments can then be used to address fundamental issues regarding the development of IgE antibodies in allergic patients, at both the molecular and structural level. Random peptide display has greatly facilitated the discovery of epitopes recognized by serum IgE antibodies from allergic patients, and it is a definitive tool for investigating the IgE-epitope interaction. Whole allergens can also be displayed on phage. Selecting IgE binding phage from diverse cDNA libraries of allergens has assisted in the identification of new allergens and provided a source of purified allergens for the diagnosis of allergic diseases. Finally, phage display of antibody fragments and random peptides is currently providing a means by which the IgE antibody can be targeted as a potential treatment for allergy. This review highlights several studies which have utilized phage display methodology in the area of allergy research, and it discusses how the therapeutic potential of this approach may be exploited.

INTRODUCTION

Allergic diseases such as asthma, eczema, rhinitis, dermatitis, conjunctivitis and anaphylactic shock appear to be on the increase in the western world [1]. These disorders are caused by the interaction between IgE and innocuous protein antigens known as allergens. Allergens are produced from a wide range of sources including animal danders, dust mites, pollens, and fungal spores. IgE is produced by B cells and the Fc portion of this antibody is designed to bind to the high affinity receptor for IgE (FcεRI) present on mast cells in tissues or basophils in the bloodstream. Once bound, IgE persists on the cell surface for many months and upon contact with allergen, a number of mediators synthesized by the effector cells are released. Cell bound IgE is crosslinked by epitopes or determinants upon the allergen which then result in aggregation of the FcεRI. The aggregation of receptors initiates an enzyme-mediated cascade of events which ultimately leads to the degranulation of prestored mediators such as histamine, and the consequent immediate clinical symptoms of allergy (reviewed in [2]).

Mast cell and basophil activation causes synthesis of a number of factors responsible for the ongoing allergic symptoms, such as prostaglandins and leukotrienes [3]. These cells also produce interleukins-4 and 13 which mediate class switching to IgE in B cells (reviewed in [4]). The process and consequences of mast cell degranulation is

depicted in (Fig. 1). Once initiated it is difficult to interrupt this network and the treatment of allergic disorders has therefore focused on preventing the initial IgE-allergen interaction. These treatments include desensitization therapy [5] which aims to induce the production of IgG antibodies which block the interaction between IgE and allergen.

Elucidation of the molecular and structural factors which govern the IgE-allergen interaction has also been a focus of intense research efforts over the past few years [6-14]. Many technical problems are associated with the characterization of IgE antibodies in allergic individuals and the identification of their epitopes. The display of antibody fragments and antigens or peptides on filamentous phage [15] has greatly assisted in the investigation of all manner of antibody-antigen interactions, including those in autoimmunity [16-18], cancer [19, 20] and medical microbiology [21, 22]. This approach is now also proving useful in the area of allergy research, since it has allowed the isolation of IgE antibodies that are present in allergic sera at concentrations of only nanogram quantities [3]. It is also leading to the characterization of new allergens as well as the epitopes recognized by IgE. More recently, phage display has been used to produce anti-IgE reagents and has been used to design and generate potential therapeutic interventions for allergic disease.

PHAGE DISPLAY TECHNOLOGY

Phage Display Methodology - an Overview

The display of antibody fragments and peptides on the surface of fd filamentous phage is a relatively new technique

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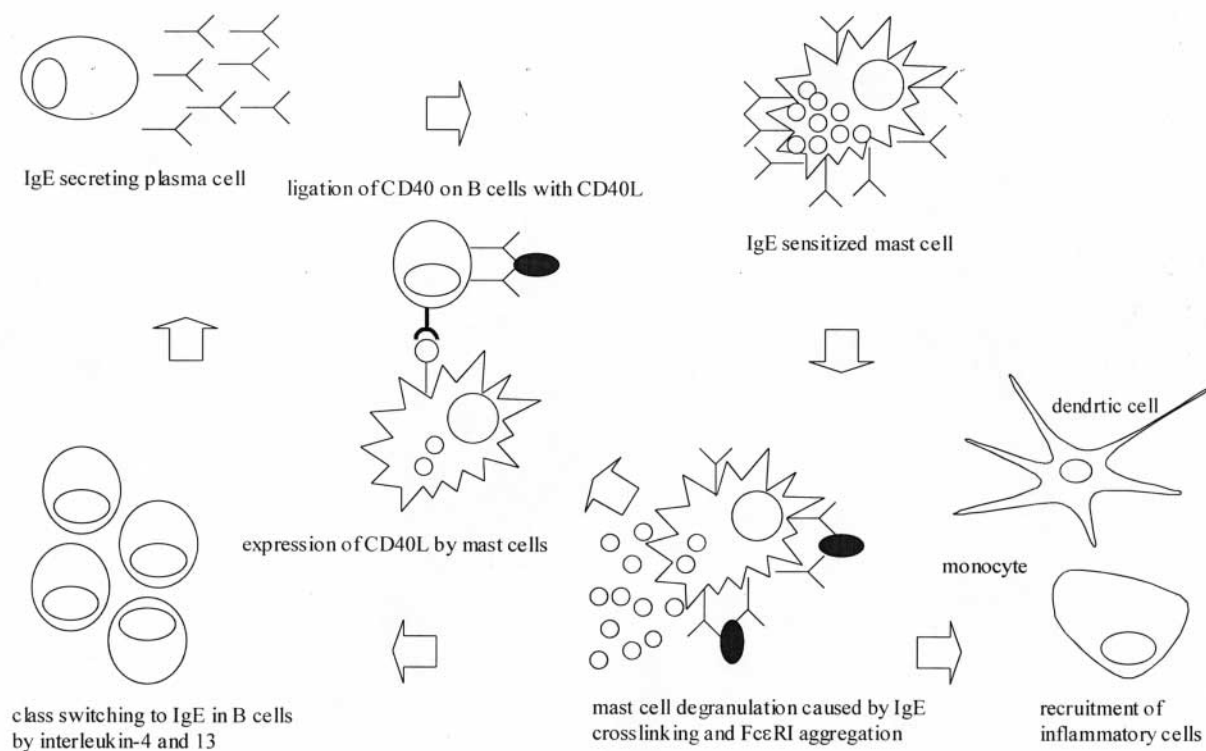


Fig. (1).

Fig. (1). The process and consequences of mast cell degranulation. IgE antibodies become bound to mast cells by FcεRI. Crosslinking of mast cell bound IgE by allergen results in FcεRI aggregation and degranulation of the mast cell. Preformed mediators are responsible for the immediate effects of allergies, while synthesis of interleukin-4 and 13 cause class switching to IgE in B cells. Other mediators may recruit inflammatory cells such as dendritic cells and monocytes. Mast cells may also provide CD40 ligand (CD40 L) required for B cell activation.

that allows the study of protein-protein interactions and greatly assists in the isolation of ligands specific for a selector molecule (reviewed in [23-27]). The technology is based on phage genetics and manipulation of the *E. coli*-fd filamentous phage lifecycle. Through this manipulation it is possible to design powerful selection strategies whereby specific phage antibody or peptide fusions are selected with a known molecule. Enrichment is achieved by repeated rounds of capture and propagation in *E. coli*.

Complete or partial genes encoding antibody or antigens can be assembled by cloning into plasmid vectors and introduction into *E. coli* producing "libraries" or "repertoires" of different genes [28-34]. For antibodies, heavy and light chain variable (V) genes are amplified by PCR, cut with restriction enzymes and cloned into a vector using DNA ligase. For peptides, it is possible to design a library based completely on random sequences [28, 30], or to create libraries of whole antigens from their cDNA [35]. Alternatively gene fragment libraries [29] can be constructed whereby the sequence of a motif, fragment or individual domain of a protein antigen can be amplified using polymerase chain reaction (PCR), and cloned.

Phage display vectors can be phagemids or phage vectors. Phagemid vectors have both an fd origin of DNA replication and also a plasmid (colE) origin of replication. The phage origin allows packaging of ssDNA into phage particles following superinfection with a helper phage, whereas the plasmid origin permits replication as a ds plasmid [36]. Both vectors also contain a phage structural gene that is the site for cloning the peptide, antigen or antibody DNA. Depending upon the vector used there is generally the choice of cloning into one of two phage structural genes. It is possible to express the cloned genes upon the surface of the tail protein glycoprotein 3 (g3p) which is present as 3-5 copies per phage. Antibody or peptide DNA are cloned either at the region coding for the N terminal domain of g3p [28, 30, 33, 37], alternatively this region may be excised from the vector and the antibody or peptide DNA cloned at the sequence coding for the second domain of g3p [38]. Bacteria electroporated with an antibody gene library can be super-infected with M13 helper phage, such as M13KO7. Infection is facilitated by the interaction of g3p and the *E. coli* F-pili, and infection allows transfer of the phage structural genes required for phagemid assembly and packaging into a functional virion [36]. Vectors used for the

creation of peptide libraries are often phage vectors, rather than phagemids, and they do not require superinfection with a helper phage. This process allows the cloned genes encoded by the phagemid to be expressed as an antibody fragment or peptide-phage fusion protein. When using vectors that make use of the truncated gene for g3p [38], the g3p fusion protein is no longer infective. Functional g3p is however encoded by the helper phage genome. Display using the truncated form of g3p often results in monovalent display, and this is achieved by phagemid systems, in which the majority of the expressed g3p is of wildtype phage rather than phage of recombinant origin. The second cloning choice is the coat protein 8 (p8) [36] of which there is 2800 copies per phage [36]. Display upon this protein allows for multivalent display, and is commonly used when displaying peptides.

Foreign DNA can also be attached to a phage structural gene through covalent interactions. The pJuFo system [35] utilizes two leucine zippers, Jun and Fos which have high affinity for each other. A phage protein is attached to the cloned protein by the Jun-Fos interaction. One leucine zipper is cloned as a phage gene fusion, the other as a fusion of the cloned gene. Expression of both the phage structural gene and the cloned gene(s) allows the covalent linkage through the leucine zippers. This method can allow the cloning of larger proteins than earlier methods of display [15], as the gene of interest is not expressed as a fusion protein with the phage structural gene.

Regardless of the display system used, the resulting recombinant phage have antibody fragments or peptides on their surface and are capable of being selected by molecules such as antigen, antibody or cell surface receptors. The phage also have the necessary genetic information to behave as intact phage, and can be used to infect wildtype *E. coli* transmitting the now single stranded antibody or peptide gene library. Thus the antibody or peptide DNA is physically linked to their protein products. Selection on the basis of binding properties also selects for the genes that determine this feature.

The design of phage fusion proteins has opened up powerful selection strategies whereby specific phage can be enriched over several rounds of affinity selection on a selecting molecule followed by propagation in *E. coli* [39]. The libraries are expressed as phage-fusions, and passed over ligand which can be immobilized [32, 33, 40, 41], present upon cultured cells [42, 43] or in solution [39, 44]. After washing, the eluted phage are removed by treatment with acid, base or protease and used to infect log phase *E. coli*. The *E. coli* are then grown and manipulated to express phage fusions and the whole procedure is repeated. By this method, rare phage fusions present at a frequency of only 1/10000 clones can be selected to a molecule of interest over many generations in *E. coli* [32]. The phage display process is summarized in (Fig. 2).

Displaying Antibody Libraries on Phage

The variable regions of antibodies are encoded by genes made by the joining of one variable (V), one diversity (D) and one joining (J) gene segment for the heavy chain, and

one V and one J gene segment for the light chain. The heavy chain locus in humans has been mapped to chromosome 14 [45], and is composed of 51 functional germline V gene segments, divided into seven families (VH1-7) based upon nucleotide identity [46]. The V genes are flanked downstream by the D genes, of which there are 30 proposed segments, again divided into seven families [47] and finally there are the J gene segments numbering only six [48]. Extensive analysis has also been performed upon the κ and λ light chain loci [49-52].

The process of recombinational rearrangement [53] brings together one V, D and J gene segment for the heavy chain, and one V and J gene segment for the light chain to form a functional V gene. Diversity is essentially generated by these rearrangements, however further diversity is added by processing at each end of the recombining elements through N and P nucleotide addition, and by exonuclease activity [53]. Finally the now rearranged V gene is juxtaposed with a set of constant region exons (μ , γ , α , δ , ϵ for the heavy chain, κ or λ for the light chain) allowing expression as a functional heavy or light chain.

The knowledge of antibody V gene segments and their organization has proved invaluable in designing sequence specific primers for amplification of heavy and light chain V genes by PCR [54, 55]. The design of constant region specific primers means that rearranged V genes in association with individual isotypes can be targeted, with no contamination from other isotypes. The design of antibody V gene specific primers, cloning of these genes, and their expression in *E. coli* to produce scFv or Fab antibody fragments has been thoroughly reviewed [23, 24, 56-58].

Displaying Whole Antigen cDNA Libraries or Gene Fragment Libraries on Phage

Whole antigens or portions of an antigen can also be displayed on the surface of fd filamentous phage. This methodology involves cloning all available cDNAs produced from mRNA of an organism, such as a fungus. All cDNAs are cloned non specifically using linker oligonucleotides, and expressed on phage as a covalently attached molecule rather than gene fusion [35]. This method offers the potential to isolate ligands from the entire cDNA population secreted from an organism at a particular time.

Typical gene fragment libraries are produced when the coding sequence of an antigen, for example, is digested with DNase in such a way as to generate fragments of 50-300 bp in size. These gene fragments can then be cloned into the g3p gene in an appropriate phage vector using oligonucleotide linkers [29]. The resulting library will contain different overlapping portions of the antigen that will often be large enough to maintain some of their native three dimensional structure when displayed. Fragments of up to 100 amino acids have successfully been displayed in this way [27]. This approach has the disadvantage of being applicable only when the antigen sequence is known and available. An additional disadvantage of displaying fragments of antigens is that many will likely not fold as they comprise only parts of protein structural domains.

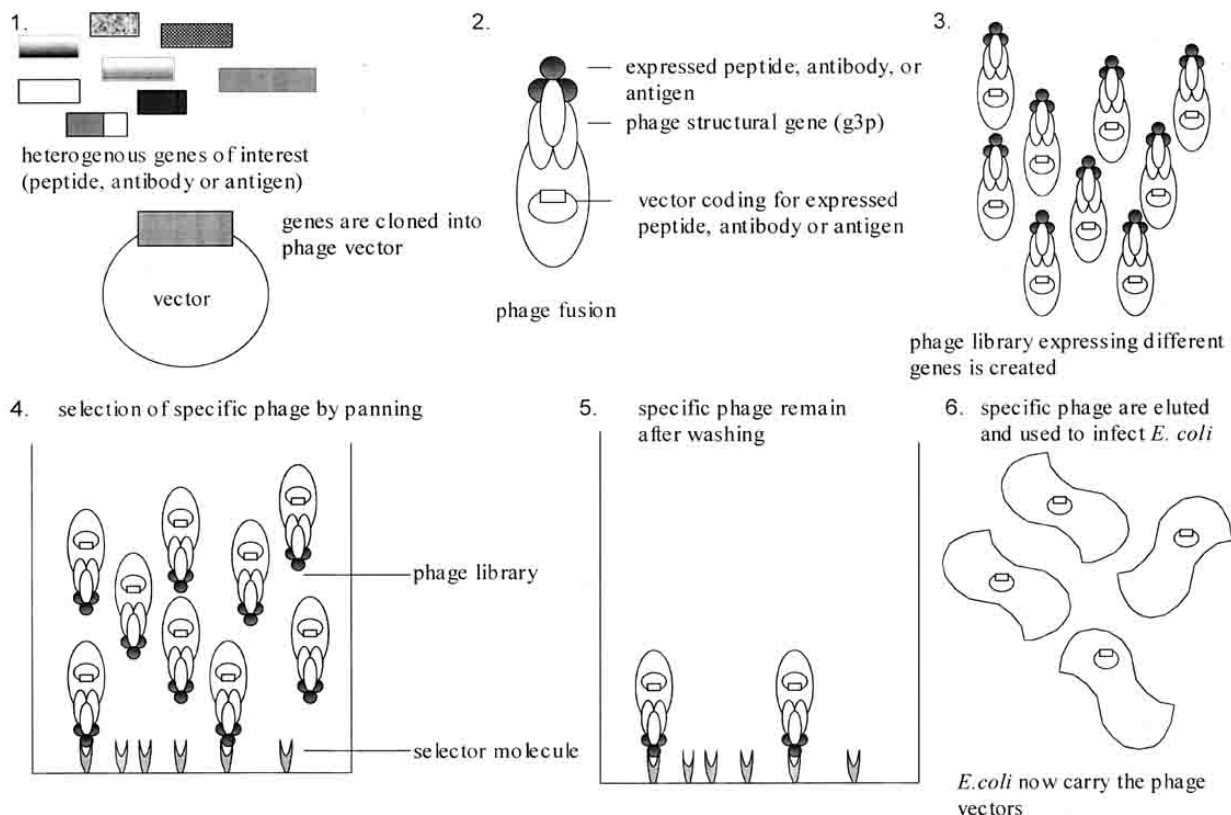


Fig. (2). Summary of the phage display approach. Genes of interest are isolated and cloned into a suitable phage display vector (1). Following expression these genes are presented as a fusion protein or covalently associate with a phage structural gene. The phage also carries the gene for the expressed protein of interest (2). Expressing the entire library produces a range of phage with different expressed proteins and hence different binding characteristics (3). Phage with specific binding properties can be selected for binding to a known molecule (4,5). Binding phage can then be amplified by infecting *E. coli*, which will now contain the genetic information for the selected protein of interest. This process is repeated enriching phage with the selectable properties (6).

Displaying Random Peptide Libraries on Phage

As an alternative to the whole cDNA or gene fragment approach, antibody-antigen interactions can be investigated using libraries of random amino acids, generated by cloning random oligonucleotides into the gene coding for g3p [30] or p8 [59]. The random sequences can range from 6-43 amino acids [28, 59-61] and can be displayed in one of two formats. Firstly, each random peptide can be displayed as a linear molecule upon the phage protein [28, 30, 60] which will assume a number of possible conformations. Thus the same peptide sequence can theoretically be presented as different conformations within the library. The second format is to conformationally constrain the peptide sequence using conserved cysteine residues encoded on either side of the peptide cloning site [62, 63]. The cysteine residues in many cases likely form a disulfide bond presenting the peptide as a loop, allowing for a more natural conformation to be assumed. Internal cysteine residues can also be designed within the peptide sequence [61] allowing the formation of internal constraints.

The purpose of the random peptide approach can be to identify a ligand which mimics the structure, part of the sequence or both of a larger proteinaceous entity such as an epitope. The identified ligand is thus known as a mimotope [62]. This approach has the advantage of being able to select for ligands when the actual sequence of the antigen is unknown [64, 65]. Although the isolated mimotopes may not be exactly like the corresponding epitope in terms of sequence or structure, they do allow for some description of the epitope to be generated.

THE IMPACT OF FILAMENTOUS PHAGE DISPLAY ON ALLERGY RESEARCH

Studies of IgE Antibodies Using Phage Display

IgE is present in the serum of atopic individuals at concentrations of only 50-300 ng/mL [3]. This is a significant impediment to studies of IgE antibodies using conventional serological techniques. Much of what is known

about IgE antibody structure (Fig. 3) has therefore been derived from studying IgE myeloma proteins [66-70]. Such studies do not however provide any information about an ongoing IgE response in an atopic individual. Unfortunately, IgE secreting B cells are infrequently found in the peripheral blood [71, 72] and large numbers are hard to obtain even from lymphoid tissue. These features have further hampered the study of IgE antibodies and basic questions such as how IgE responses develop in atopic individuals remain unanswered. For example, the clonal history of IgE producing B cells is unknown and it is unclear whether these cells are derived from class switched IgM producing cells [73, 74] or are derived from IgG producing cells [75-77]. The potential of B cells to undergo class switching from IgG to IgE is intriguing as it would imply that IgE antibodies

share the epitope specificity of IgG. The molecular features of IgE antibodies have also been poorly studied. For instance, there is very little information concerning the extent to which V gene usage and N and P nucleotide addition determines the specificity and diversity of the IgE response. It is also unclear if the IgE producing B cells have been selected by antigen and demonstrate evidence of hypermutation such as found in a conventional antigen driven response. For these reasons, the affinity of the IgE antibodies produced in allergic responses is largely unknown. While several studies [78-83] have attempted to address some of the above issues using DNA sequencing of cloned IgE V gene transcripts, they have all used randomly amplified transcripts of unknown specificity.

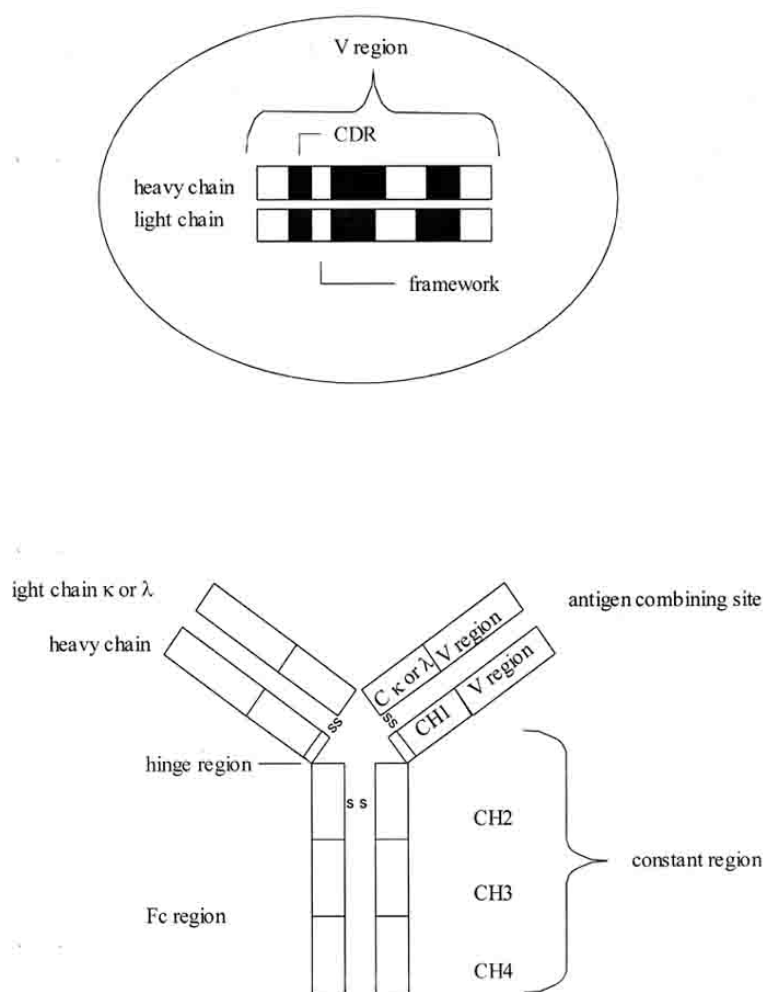


Fig. (3). Structure of an IgE antibody. The antibody consists of a heavy-light chain dimer, joined by disulphide bonding at the heavy chain. Light chains associate with each heavy chain also through disulphide bonding. There are four heavy chain constant region domains (CH1-4), and one light chain constant region, κ or λ . Constant regions CH3 and CH4 together are involved in binding to the Fc ϵ RI and Fc ϵ RII. The V regions of each heavy and light chain form the antigen binding site. Each V region is composed of three hypervariable loops or CDRs, (shown in black), supported by a β pleated sheet scaffold termed framework regions.

Allergen specific IgE antibodies have been recently isolated and characterized using phage display. As the phage fusions are physically linked to the genes determining their binding specificity, the biochemical and genetic features of allergen reactive IgE antibodies can be compared. The first IgE Fab phage display library [84] was successfully used to isolate allergen specific Fabs. In this study, Steinberger and colleagues [84] constructed a Fab IgE library of approximately 5×10^7 clones from peripheral blood collected at the height of the pollen season from a patient sensitive to grass pollens. Several IgE Fabs were isolated against the major timothy grass allergen Phl p 5. The IgE Fabs were able to be purified in milligram amounts using affinity chromatography, which clearly demonstrates that IgE Fabs can be readily expressed in *E. coli*. The IgE Fabs isolated were able to compete with serum IgE from the same patient, and cross reacted with other grass pollens such as Kentucky Bluegrass and group 5 allergens from rye grass. Based on this data, the authors believed that the isolated IgE Fabs represented the specificity of the serum IgE present in the patient. These allergen specific IgE Fabs have also been used as tools to identify cross reactive epitopes on different plant pollens. This data may explain, at the molecular level, the multiple crossreactivities of many pollen allergic individuals who recognize many allergens from different plant species [85].

Sequence analysis of the Phl p 5 specific Fabs showed a bias in heavy chain V gene usage, in that each of the four clones utilized the VH3 heavy chain gene family with different κ light chains. This would suggest that the IgE response to Phl p 5 is restricted with regard to heavy chain V gene usage, an observation that has been previously reported for IgE sequences [78, 81, 83] however not for an allergen specific IgE response.

The success of the study is surprising considering the low number of IgE secreting B cells present in peripheral blood even in atopic patients [71, 72] and the unreliability of peripheral blood lymphocytes as a source of immunoglobulin mRNA [86]. It was possible however, that as the sample was taken at the height of the pollen season, the consistent antigen stimulation increased the number of circulating IgE secreting plasma cells. This idea is supported by *in vitro* studies which show that the potential of B cells from allergic donors to produce IgE certainly increases during the pollen season [87]. The patient used in Steinberger's study [84] had detectable serum anti-pollen IgE reactivity, providing further evidence that the library was constructed at a time when there was an ongoing IgE response. As allergic responses show lower levels of serum IgE and circulating B cells than responses characterized by IgG, the generation of IgE phage antibody libraries is particularly challenging. The identification of patients with a high specific IgE response (as measured by enzyme linked immunosorbent assay (ELISA) or radioallergosorbent assay (RAST) at the time of sample harvesting may be a prerequisite for the construction of suitably enriched IgE libraries. A second problem related to the generation of quality libraries is that of accessibility to suitable clinical samples. In this regard, lymphoid tissues (tonsils, lymph nodes, bone marrow) are superior to peripheral blood [86] however atopic individuals seldom undergo invasive procedures where the above samples are

removed. The unavailability of clinical material represents a problem for the analysis of IgE antibodies particularly when studying allergies other than pollen allergy. One alternative would be to sort IgE positive B cells using flow cytometry and then stimulate these cells *in vitro* with a B cell mitogen, thus increasing the amount of IgE mRNA. There are concerns however that *in vitro* culture may affect the original diversity of the B cell population [88]. This is an additional problem to the random association of the heavy and light chain genes by the phage display process.

To date, one other IgE phage antibody library has been generated and analyzed (M. Edwards, R. Ward, A. Collins manuscript in preparation). This library was constructed from the peripheral blood of an eczema patient who had a RAST reactivity to egg proteins. The library was panned against ovomucoid, a major egg allergen [89] and twelve anti-ovomucoid IgE Fabs were isolated. The IgE Fabs had a polyreactive binding profile, binding other non self proteins, including bovine serum albumin and skim milk protein. Sequence analysis of the reactive clones revealed a restricted heavy chain V gene usage. All clones utilized the only member of the VH6 heavy chain family, a gene segment that has been previously associated with B-1 derived antibodies [90, 91]. Sequence analysis of clones randomly selected from the IgE library revealed additional evidence that suggested the B-1 cells may be a source of IgE. Evidence included a relative paucity of mutations, short complementarity determining region (CDR) 3, few N additions at the D-J junction and the predominant usage of the D7-27 D gene segment. All of these features are frequently observed in antibodies derived from B-1 cells [90, 92-95]. B-1 cells are accepted as the source of natural or polyreactive antibodies [96] and this would explain the polyreactive binding profile of the isolated IgE Fabs. Our study suggests that IgE antibodies can arise in a manner analogous to natural antibodies rather than by selection in an ongoing antigen driven immune response.

The above examples demonstrate the potential use of phage display in the study of IgE antibodies. Although there are some technical difficulties, phage display is really the only method currently available for the study and isolation of specific IgE produced in the allergic responses of atopic individuals. Refinements in existing technologies will undoubtedly overcome the technical problems and the application of phage display will become more widely accessible in the field of allergic research. The application of phage display to IgE analysis is sure to provide continued insights into the intricacies of IgE antibody generation at the molecular, genetic and cellular level.

Anti-Allergen Fabs as Potential Therapeutic Agents

As IgE antibody fragments do not contain C ϵ 3 or C ϵ 4 constant region domains, they will not elicit the effector functions of IgE, such as binding to Fc ϵ RI and Fc ϵ RII on basophils and mast cells. This makes it therapeutically attractive to use these recombinant IgE antibody fragments to block the binding of serum IgE. Although the potential of allergen specific Fabs as therapeutics has been reviewed elsewhere [97], some of the original studies regarding the use and isolation of allergen specific Fabs by phage display were

not discussed in the above review and are therefore presented in the following section.

A synthetic combinatorial library, in which the heavy chain CDR3 region was composed of randomized sequences [98] has been used to produce specific Fabs to the rye grass allergen Lol p II [99]. Thirteen specific clones were identified by panning against immobilized Lol p II and four of these were selected for further analysis. DNA sequencing of the cloned V genes demonstrated that all clones exhibited the same λ light chain sequence, and identical heavy chains (VH1 family), with one clone's heavy chain differing from the other heavy chains by only two amino acids. The clone with the highest affinity ($2.63 \times 10^{-9} \text{ M}^{-1}$) was shown to inhibit the binding of diluted rye grass allergic serum IgE to immobilized Lol p II. This suggests the epitope specificity of the recombinant Fab mirrored that of the serum IgE. Epitope mapping using overlapping peptides that spanned the entire length of Lol p II indicated the epitope of the Fab was between residues 39-51. In a further experiment, histamine release from basophils was inhibited by including Fab in a bioassay using whole blood from a rye grass allergic donor Lol p II. This would suggest that the Fab selected from the synthetic phage antibody library was capable of interfering with IgE-Lol p II interaction, thereby blocking histamine release from human basophils.

To determine its therapeutic potential, the anti-Phl p 5 Fab has been expressed as a whole human IgG1 molecule [100]. Although this antibody was not able to block the binding of allergic serum IgE to the Phl p 5 allergen, such antibodies may still be clinically useful. Human [101] and rat mast cells [102] also contain low affinity receptors for IgG (Fc γ RII and Fc γ RIII). In animal models, formation of cell bound complexes of IgG and allergen has been shown to inhibit *in vitro* mast cell activation when these immune complexes are crosslinked with cell bound IgE [103, 104]. To our knowledge, this function of allergen specific IgG has not been evaluated in human patients. Allergen specific Fabs isolated by phage display and expressed as human IgG1 molecules may therefore have therapeutic effects such as preventing mast cell and basophil degranulation through a receptor mediated inhibition mechanism rather than as a blocking antibody.

The area of allergy research has not fully taken advantage of the many synthetic sources of recombinant antibodies [98, 105-107]. These libraries containing antibody sequences with synthetic or randomized CDRs, could potentially contain a wealth of anti-allergen antibodies. The antibodies could be useful as blocking antibodies, as described above. These libraries have provided antibodies to cancer antigens [105, 106] hormones [106, 107] and proteins of the immune system [105, 107]. The lack of studies using these libraries as a source of anti-allergen antibodies probably reflects the fact that the interest in allergens has always been a priority.

Analysis of the IgE-Epitope Interaction

While the IgE antibody-allergen interaction is crucial for the initiation of the cellular response in allergic reactions (reviewed in [2, 3]) it is not well understood. In the past, a

number of techniques have been used to characterize the epitopes recognized by IgE. These include the assessment of IgE binding after treatment of whole native allergens with chemicals or heat treatment that disturb the three dimensional structure of the allergen [6-8]. In addition, epitopes have been mapped using short overlapping peptide sequences that span the entire length of the allergen [9-14]. IgE epitopes on allergens have also been compared with the specificity of monoclonal antibodies (mAbs) raised in immunized mice [108-114]. Despite modest success using these approaches, they all have significant disadvantages. For example, although treatment of allergens with structure modifying agents has provided some insights into the importance of the three dimensional structure of allergens for IgE binding, it does not allow the determination of the definitive structure or sequence of the epitopes. The use of short overlapping synthetic peptides may give misleading results, as peptides often assume random conformations in solution, and do not fully represent the native allergen. For these reasons this method of identifying B cell epitopes has often been criticized [115, 116]. Raising mAbs to allergens in mice has also been used however this approach may not identify all possible IgE binding epitopes, but rather the immunodominant epitopes in inbred mice hyperimmunised with allergen.

Phage display offers many advantages over the techniques described above. Random peptide libraries may potentially cover the sequence or structural diversity of many allergens. They are self renewing, inexpensive and information can be generated within days. Peptide epitopes can also be offered in constrained or unconstrained forms so that mimotopes of discontinuous epitopes can be identified [21, 63]. Once the mimotope is identified, it can be expressed as a free protein in *E. coli* and purified from supernatant providing a ready supply of the mimotope for further studies. Phage display thus offers an additional approach to the investigation of IgE epitopes and also provides a way of generating a pure source of epitopes. In the following section several studies where phage display was used to identify IgE-binding epitopes in allergens will be discussed.

Leitner and others [117] have used constrained and unconstrained 9mer peptide libraries displayed on phage to investigate IgE binding to the plant allergen profilin. The libraries were mixed and panned against affinity purified, profilin specific IgE/IgG antibodies from the serum of allergic patients. Five out of eight peptides isolated from panning contained the sequence CAISGGYPVC, the cysteine residues indicating that the peptides had been selected from the constrained rather than the linear library. However the peptide sequence did not match a motif within the known crystal structure of birch profilin [118]. The constrained peptide was therefore thought to mimic a part of the conformation of a B cell epitope present in birch profilin. This theory was proven by showing that the synthetic peptide could inhibit the binding of birch pollen, mugwort pollen and celery tuber profilin to the purified IgE/IgG fraction as well as to sera obtained from profilin allergic patients.

In another study [119], constrained and unconstrained 9mer random peptide libraries were used to identify the mimotopes to purified IgE in individuals allergic to birch

pollen. Panning the pooled libraries generated six separate sequences, four of which were derived from the constrained library. None of these sequences matched the primary amino acid sequence of Bet v 1, the major birch pollen allergen. A computer aided algorithm was employed to compare mimotopes with the allergen sequence based on amino acids with similar chemical properties. Using this approach, the isolated mimotopes were observed to match two distinct regions in the Bet v 1 sequence, thus indicating it constituted a large discontinuous epitope. One mimotope, designated Bet min E was chosen for further analysis, as it was the dominant clone in the final round of panning. Phage expressing this sequence were used to immunize mice. The mice exhibited an IgE response that was shown to bind native Bet v 1, and the mimotope also inhibited murine IgG binding to native Bet v 1. Murine IgG also inhibited binding of the purified IgE used in panning to the mimotope. Based on these findings, the mimotope was thought to represent part of an actual epitope in the native Bet v 1 which is recognized by IgE in birch pollen allergic patients. The authors suggest that mimotopes displayed on phage could possibly be used as an allergy treatment since IgE binding mimotopes would generate an IgG response in allergic patients. While this is true for laboratory mice, it is unknown whether or not such an immunization strategy would generate blocking IgG antibodies in allergic humans.

Phage display has also been used to identify IgE epitopes on Der p 1, a cysteine protease and important allergen of dust mite [120]. The study utilized the mouse monoclonal 2C7, an antibody which binds to the same epitope on Der p 1 as that recognized by a large proportion of allergic human serum IgE [121]. The mAb 2C7 was used to select phage present in an unconstrained 15mer peptide library, expressed on p8. Several clones were isolated, each having the consensus sequence DXXXR or KXXGR. The consensus sequences corresponded well to the region of leu147-gln160 present in the crystal structure of Der p 1 [122] as part of a β pleated sheet. Similar structures were then found on the related molecules papain, chymopapain and actinidin which are also cysteine proteases. Two phage clones having the peptide sequences of TPLCDYAAARVGACG and NCLSSDEPLH IRWCQ inhibited the binding of purified Der p 1 to the mAb 2C7 in an ELISA. As both clones contain cysteine residues within their sequence, it was thought that this assisted in Der p1 binding. Other phage clones with internal cysteines could not inhibit binding of Der p 1 to 2C7.

The identification of IgE binding epitopes using random peptide libraries has clearly provided insight into the structural motifs or regions of allergens which are important in allergic patients. In addition, these mimotopes may have therapeutic potential since they can only bind to one of the antigen binding sites on IgE. The mimotopes could therefore interfere between the binding of IgE and native allergens by binding but not crosslinking cell bound IgE. As the cell bound IgE would not be crosslinked, degranulation of mast cells or basophils would not occur. Ongoing work is required to determine the full clinical potential of this approach.

Isolation of New Allergens

New protein allergens have been characterized recently using phage display. This has particularly found application

in the realm of fungal allergens. The number of fungal species occurring in the human environment has been estimated to be at least 100 000 [123] and many of their expressed proteins can potentially act as allergens. Investigation of this issue is hampered however by the unavailability of well defined allergen preparations [124]. Phage display is having a major impact in this area of allergy research, as it has facilitated the isolation of new fungal allergens and also the production of pure, recombinant allergens. The latter is mandatory for the ongoing diagnosis of fungal related allergic disorders.

Phage display was recently used to identify IgE binding allergens produced by *Malassezia furfur* [125]. Complementary DNA from the organism was cloned into the pJuFo vector, allowing construction of a library expressing whole native allergens on phage surfaces. The library was panned upon serum obtained from an atopic dermatitis patient having a high RAST to *M. furfur* extract. Phage were isolated and nucleotide sequencing revealed five different sequences. Two of the inserts, (designated Mal f 5 and Mal f 6), had sequence identity with other known allergens Mal f 2, Mal f 3 and (cyclophilin) of *Schizosaccharomyces pombe* respectively. The other three sequences (designated MF 7,8,9) showed no similarity with known proteins. The five clones produced from the panning experiment were purified from *E. coli* and shown to be reactive to a varying degree with serum IgE obtained from twenty five patients having positive RAST to *M. furfur* extract. None of the isolated proteins were recognized by RAST negative sera or by myeloma IgE protein. As the proteins MF 7, 8 and 9 were recognized by IgE, they may represent new allergens of *M. furfur*.

New allergens of *Aspergillus fumigatus* have also been isolated using the phage display of whole allergens [126]. A new allergen was isolated from *A. fumigatus* phage display library made using the pJuFo vector system [35]. Isolated by panning on the serum of *A. fumigatus* sensitized individuals, the reactive clone contained an insert of 616 bp containing a start and stop codon which predicted a protein of 168 amino acids. The new protein (designated Asp f 3) demonstrated identity with two peroxisomal membrane proteins (PMP-A and PMP-B) of *Candida boidinii*. In a second series of experiments, PMP-A and PMP-B were cloned from genomic DNA sequences and recombinant protein prepared in *E. coli* along with recombinant Asp f 3. Western blots demonstrated that each of the recombinant proteins could be recognized by IgE present in the sera obtained from patients sensitized to *A. fumigatus*. Both *Candida* proteins were then shown to inhibit Asp f 3 binding to serum obtained from Asp f 3 sensitized patients. This data suggested that the three proteins may have similar IgE binding epitopes, and was important as it was the first study to show IgE cross reactivity between fully characterized fungal products. Furthermore, it demonstrated that the isolation and characterization of a new allergen (Asp f 3) by phage display led to the identification of two proteins (PMP-A and PMP-B), that had been previously unreported as allergens.

The diagnosis of sensitivity to fungal species remains an ongoing problem as there are few purified fungal allergens. This problem is compounded in cystic fibrosis (CF) patients,

who sometimes develop allergic bronchopulmonary aspergillosis (ABPA). In ABPA, infection with *A. fumigatus* leads to eosinophilic and lymphocytic infiltrates in the airways. The infected patients develop increased serum IgE levels and precipitins containing *A. fumigatus* allergens in serum [127]. Discrimination between CF patients with ABPA and CF patients with allergy is difficult [124, 128] as skin and RAST tests do not distinguish one disease from the other. Discrimination is however crucial, as the two diseases require different treatments.

In a recent study [129] a panel of allergens was used to discriminate between CF patients with ABPA and those with allergic reactions to *A. fumigatus* allergens. The allergens were derived using a phage display library expressing *A. fumigatus* allergens [35, 130]. The study [129] tested four allergens (Asp f 1, Asp f 3, Asp f 4 and Asp f 6) for binding to serum from fifty CF patients with either ABPA, sensitized to *A. fumigatus* allergens, or having no sensitivity to *A. fumigatus* or ABPA. IgE from both ABPA and allergic patients recognized Asp f 1 and Asp f 3 by ELISA but not CF patients without either disorder. In contrast, reactivity to Asp f 4 and Asp f 6 were solely recognized by the serum IgE of patients with ABPA. A reaction to Asp f 4 and Asp f 6 could therefore be used to discriminate between ABPA and sensitization to *A. fumigatus* in CF patients.

Isolation and Characterization of Anti-IgE Antibodies Using Phage Display

The presence of autoantibodies specific for IgE in atopic patients [131, 132] and normals [133] is well documented. Until recently, these antibodies which may serve as potential therapeutics have not been able to be isolated or extensively studied. Phage display provides a means for the isolation and further characterization of these antibodies.

In 1994 Vogel and colleagues [134] isolated several anti-IgE antibodies from a phage display library made from peripheral blood B cells of an atopic donor. Anti-constant region and anti-idiotypic antibodies were both isolated from the library, thus demonstrating for the first time that not only anti-idiotypic but anti-constant region domain antibodies were produced against IgE in atopic individuals.

Further examination of the anti-constant region Fabs demonstrated a binding specificity to the Cε2 domain of human IgE. As Cε2 specific monoclonals have been shown to enhance IgE binding to the low affinity receptor FcεRII (CD23) [135], such Fabs could potentially exacerbate the atopic condition. This was an important discovery, as it demonstrated that potentially harmful anti-IgE antibodies

could be made in atopic individuals. A greater understanding and ability to control the anti-IgE response is needed before anti-IgE treatment could be considered as a therapy for allergy.

Development of an Allergy Vaccine Using Phage Display

A range of anti-IgE mAbs are presently under investigation as therapies for allergic disease and two of these are currently in clinical trials [136, 137] (Table 1). These latter therapies are humanised murine antibodies [136] or chimeric mouse-human antibodies [137] which are designed to interfere with the IgE-FcεRI interaction. In a recent article, Stadler and colleagues [138] have discussed the disadvantages associated with such a strategy. The anti-IgE monoclonals need to be administered at concentrations in excess of IgE, necessitating repeated and ongoing therapy.

As an alternative, Stadler and colleagues have suggested that the anti-IgE response can be harnessed and redirected, to produce defined anti-IgE antibodies that are able to control the allergic response in atopic individuals [139]. In support of this, the group used phage display to produce mimotopes of a murine mAb (BSW17) that interferes with the IgE-FcεRI interaction. Importantly, the use of these mimotopes as an immunogen have been shown to generate an anti-IgE response in animal models. Development of these mimotopes were greatly facilitated by phage display techniques and provide a good model for the design of an anti-IgE vaccine. A summary of this work is provided in (Table 2).

BSW17 [140] is a murine mAb that can interfere with IgE-FcεRI binding, inhibit human IgE synthesis *in vitro* [141] and can bind and remove IgE residing on the surface of effector cells without causing their degranulation [142]. These features make it attractive as a therapy for allergy. The binding of BSW17 to human IgE was first investigated using constant region domain specific monoclonals and recombinant Cε domains [135]. As a result of these studies it was thought that BSW17 recognizes the Cε4 domain exclusively.

However, the binding specificity of BSW17 as determined by phage display of random peptides generated slightly different data. Binding specificity was investigated using constrained and unconstrained 9mer random peptide libraries and a gene fragment library of human ε constant regions displayed on phage [143]. Specific peptides were isolated from the constrained 9mer random library that could bind BSW17, but not other murine anti-human IgE mAbs. All BSW17 binding clones consisted of two amino terminal proximal amino acids that had a polar charge, followed by

Table 1. Summary of Anti-IgE Therapies Currently in Clinical or Pre Clinical Trials

Agent	Function	Clinical Trial	Reference
Humanized mouse anti-IgE mAb	Interfere with IgE-FcεRI binding	Phase III	[136]
Chimeric mouse-human anti-IgE mAb	Interfere with IgE-FcεRI binding, lysis of IgE expressing B cells.	Phase III	[137]
Soluble α chain of FcεRI	Interfere with IgE-FcεRI binding	Pre clinical	[149]

Table 2. Summary of the Key Milestones in the Development of an Anti-IgE Vaccine Using the mAb BSW17

Reagent	Significance	Reference
IgE mimotopes	Mimotopes inhibit BSW17 binding to IgE, and can generate an anti-IgE response in rabbits.	
Cε3 and Cε4 mimotopes	Demonstrated that BSW17 recognises both Cε3 and Cε4 domains of human IgE. This helped explain why BSW17 is nonanaphylactogenic and can remove FcεRI bound IgE	[144]
Cε3 and Cε4 mimotopes	Can prevent passive cutaneous anaphylaxis in rhesus monkeys	[138]
Anti-BSW17 idiotypic antibodies	The V regions of heavy and light chain of these antibodies contained sequence identity with the Cε3 region of human IgE.	[146]
Anti-BSW17 idiotypic antibodies	Anti-idiotypic antibodies could generate an immune response in rabbits which was capable of inhibiting IgE binding to FcεRI, thus mimicking the original properties of BSW17.	[146]
Cε3 and Cε4 mimotopes, and anti-idiotypic antibodies	Function in a gastric simulation test when expressed as a p8 fusion. Produced an anti-IgE antibody response when used to immunize mice orally. Evidence of phage replication found in mouse faeces.	[147]

three uncharged, polar amino acids. This motif also contained two amino acids at the carboxyl terminus that were always tryptophan and valine. Interestingly, the sequences of the BSW17 binders were not homologous to the sequence of the human IgE constant region, suggesting that the structure or chemistry of the mimotopes merely mimicked a region of human IgE. Two mimotopes were able to inhibit the binding of BSW17 to human IgE, and were found to be immunogenic when used in rabbits as phage fusions. Serum obtained from the immunized rabbits demonstrated an anti-IgE response, and could inhibit BSW17 binding to human IgE.

A motif in Cε4 was identified whose amino acids had similar chemistry to that of the mimotopes [144]. A gene fragment library of the ε constant regions was then created, such that phage either expressed one or several whole ε constant region domains upon their surface. This was used as a tool for further exploring the binding specificity of BSW17. BSW17 was seen to bind phage expressing either Cε1-Cε4 and Cε4 alone, but not to phage expressing the other domains, suggesting the epitope is actually within Cε4. Further experiments, using random peptide libraries isolated several BSW17 binding clones having sequence identity with the Cε3 domain. Taken together, the authors believed that the epitope for BSW17 included residues within both the Cε3 and Cε4 domains.

To further define the BSW17 epitope, synthetic peptides of the Cε4 epitope and a 14mer sequence composing the carboxyl terminal amino acids of the Cε3 domain were made and it was shown that BSW17 was able to recognize both peptides. Finally two mimotopes, one having structural homology with the Cε4 domain (Cε4 mimotope) and one having sequence identity with the Cε3 domain (Cε3 mimotope) could inhibit binding of each other to BSW17 when coupled to keyhole limpet haemocyanin. This indicates that BSW17 recognizes two distinct sequences. Taken together this data indicates that the mAb BSW17 either recognizes a large discontinuous epitope spanning both Cε3 and Cε4 domains, or it recognizes two distinct epitopes on the same molecule. The characterization of the BSW17-IgE interaction using phage display also explains the unique biological properties of this antibody. The region within Cε4 that is recognized by BSW17 has been postulated to be

involved in mast cell triggering [145]. Binding of BSW17 to IgE on mast cells would prevent the Cε4 domain associating with the FcεRI, and thus would explain why BSW17 is anaphylactogenic. Also, binding to Cε3 may explain how BSW17 can remove IgE from FcεRI upon cells as this region is important for IgE-FcεRI binding [3].

The Cε4 and Cε3 mimotopes described have also been reported to induce a protective anti-IgE response in rhesus monkeys [138]. As human IgE can not bind to the effector cells of rodents, rhesus monkeys were immunized with the phage-displayed mimotopes and they were shown to develop a strong anti-IgE response. The monkeys were then passively sensitized with a human chimeric antibody to nitrophenylacetate-bovine serum albumin. Injection with the antigen caused passive cutaneous anaphylaxis in control monkeys immunized only with antigen, however no reaction was seen in monkeys pre-treated with either of the two IgE mimotopes. This data shows that pre-treatment with mimotopes results in the development of neutralizing anti-IgE antibodies which are capable of interfering with IgE-FcεRI binding. This immunization strategy may therefore offer the same protective response in humans.

Furthermore, the potential of anti-BSW17 Fabs to act as an anti-idiotypic vaccine has recently been proposed by Vogel et al. [146]. Human Fab libraries were constructed from atopic and healthy human donors, as well as purified B cells from the tonsils of children. Following panning against BSW17, eight reactive clones were isolated. Importantly, the binding of these clones to BSW17, could be inhibited by both the Cε3 and Cε4 mimotopes. Sequencing the cloned V genes demonstrated that only two distinct heavy and light chain combinations were isolated. The heavy chains were derived from families VH3 and VH4, while the light chains were also derived from two families, VK1 and VK1. The soluble Fabs of the two unique clones could inhibit the binding of BSW17 to IgE bound upon CHO cells expressing human FcεRI, suggesting that the anti-idiotype Fabs recognize an epitope identical to that of BSW17.

The epitopes of IgE mimicked by the anti-idiotype Fabs were discovered by sequence comparisons of each Fab with the IgE constant region sequence. The CDR1 and FR2

regions of the heavy chains of both Fabs mimicked the Cε3 domain of IgE. The area comprising 355-383 was generally recognized by the two Fabs, an area which also had identity with the Cε3 mimotope. Further mimicry could also be found between the heavy and light chains of the Fabs and the Cε3 domain sequence, suggesting that the Fabs may recognize a larger area of IgE than BSW17.

As the anti-idiotypic Fabs demonstrated structural identity with human IgE, their ability to generate an anti-human IgE response was tested in rabbits. After immunization with either Fab, the rabbit sera was capable of inhibiting IgE-binding to its Fc receptor. The affinity of each polyclonal sera was also shown to be very high (K_d of 6×10^{-9} and $4 \times 10^{-10} \text{ M}^{-1}$).

The results demonstrate that the anti-idiotypic antibodies accurately reflect the internal image of IgE and can be used as an immunogen in experimental animals. The anti-anti idiotypic antibodies produced can thus recognize IgE and retain the original properties of BSW17. As compared with the use of BSW17 as a therapeutic agent, anti-idiotypic antibodies have the advantage of being completely human, and may prove to be better tolerated than synthetic peptides coupled to immunogens. The generation of anti-idiotypic antibodies is presented in (Fig. 4).

The feasibility of IgE derived peptide mimotopes, and anti-idiotypic human Fabs to act as an oral vaccine was recently evaluated [147]. The success of an oral vaccine in

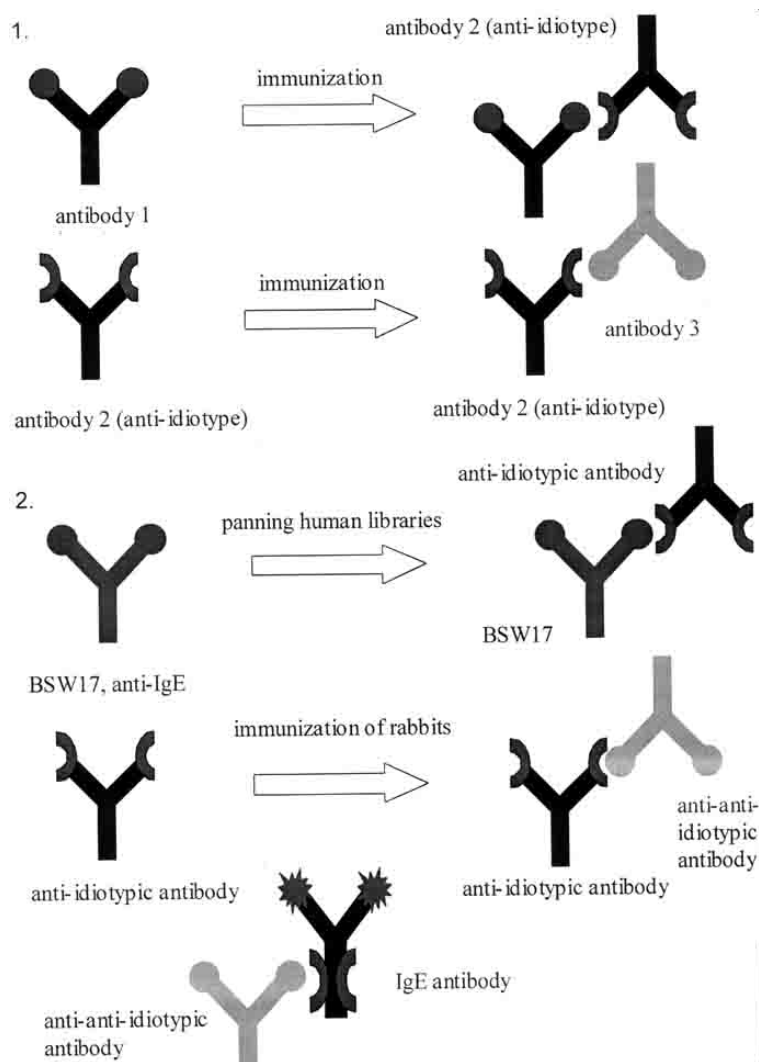


Fig. (4). Summary of the generation of anti-idiotypic antibodies using phage display. The original idiotype network hypothesis of Jerne [148] states that immunization of antibody 1 generates an antibody specific for the antibody's binding site (antibody 2). Upon a further response to antibody 2, another antibody (antibody 3) is generated specific to the binding site of antibody 2. Antibody 1 and antibody 3 therefore have the same binding site (1). Vogel [146] generated anti-BSW17 Fabs from a human phage display library. When used to immunize rabbits these Fabs generated anti-anti-idiotypic antibodies capable of recognizing human IgE and had the same specificity as BSW17 (2).

part relies on the ability of the immunogen to withstand the extreme environment of the stomach. With this in mind, a gastric simulation assay was used to test the infectivity of the phage particles and also the integrity of the expressed proteins. Following incubation in pepsin and low pH, phage infectivity and IgE binding was monitored over time. The results indicated that most phage preparations remained infective for up to 60 minutes. Results also indicated that mimotopes or Fabs expressed upon g3p were degraded after only 1 minute. In contrast, phage expressing mimotopes on p8, or purified p8-mimotope fusions were still functional up to 60 minutes.

Phage expressing the Cε3 and Cε4 mimotopes as well as anti-idiotypic Fabs on either g3p or p8 were then used to immunize mice orally. All phage preparations induced an anti-phage antibody response, as well as an anti-IgE response. Sera from all the phage preparations could also inhibit binding of BSW17 to IgE, indicating the immune sera contained antibodies of the same specificity as BSW17. Finally, replication of phage DNA was shown to be viable up to 10 days after oral immunization. This suggests that the recombinant phage infected resident *E. coli* and transiently persisted in the gastrointestinal tract.

There are distinct disadvantages associated with passive immunization with chimeric anti-IgE antibodies making these reagents of limited use. There are also inherent problems controlling the anti-IgE response. With the diverse range of anti-IgE antibodies with differing biological functions that can be generated in the human anti-IgE response, immunization with whole IgE is not feasible. Immunization of mimotopes as phage fusions or the design of Cε related peptides has potential as an anti-IgE vaccine, as demonstrated by the above work. The ability to identify and isolate peptide sequences that can act as an epitope using phage display has opened up new opportunities for anti-IgE therapy.

CONCLUSION

Phage display has provided important insights into the structure of IgE as well as epitope specificity of this antibody. While there are few studies which have used phage display to characterize IgE antibodies, this largely reflects the technical difficulties relating to sourcing IgE mRNA. These problems have meant that greater attention has been focused on the allergens rather than the IgE molecule itself. Phage display has been used to isolate new allergens and to define the structure and composition of IgE binding epitopes. Experimental data has now provided *in vitro* evidence that mimotopes isolated by phage display may serve as an intervention for allergies by interfering with allergen-IgE binding. One of the most promising approaches has been the generation of an experimental allergy vaccine, based upon mimotopes and anti-idiotypic antibody fragments displayed on phage. These therapeutic agents have advantages over current therapies as they generate a lasting anti-IgE response, and they can be used orally. Undoubtedly the next few years will see the application of this approach in human studies. If successful, it will offer a new modality of treatment for allergic disorders.

ABBREVIATIONS

V	=	Variable
D	=	Diversity
J	=	Joining
PCR	=	Polymerase chain reaction
g3p	=	Glycoprotein 3
p8	=	Protein 8
ELISA	=	Enzyme linked immunosorbent assay
RAST	=	Radioallergosorbent assay
CDR	=	Complementarity determining region
mAb	=	Monoclonal antibody
PMP	=	Perioxisomal membrane protein
CF	=	Cystic fibrosis
ABPA	=	Allergic bronchopulmonary aspergillosis
CD40L	=	CD40 ligand

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