

Allergen mimotopes

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Abstract

The causative treatment of type I allergies is a long pursued goal in immunology. To design safe and efficient vaccine preparations, the interaction of the allergen and the symptom-inducing IgE antibodies still needs to be better understood. In this article, we describe the use of the phage display technique in allergy. It yields epitope mimics, so-called mimotopes, which can be employed for both, investigation of allergen–IgE interactions and definition of safe novel vaccines.

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1. Introduction

Type I allergy, an immune disorder that is based on the production of Immunoglobulin E (IgE) against per se innocuous antigens (i.e., allergens) [1,2], represents a health problem for about 25% of the population in industrialized countries. Cross-linking of effector cell-bound IgE by allergens results in the release of biological mediators (as histamine and leukotrienes) [3,4], leading to symptoms like allergic rhinitis, conjunctivitis, atopic dermatitis, allergic asthma, and, in severe cases, anaphylactic shock. Current clinical strategies for allergy treatment involve allergen avoidance by patients, pharmacotherapy, or immunotherapy. Only the third strategy has the potential to provide a causative treatment, but its mechanisms of action are not yet fully elucidated. Better understanding of the allergen–antibody interaction and safe preparations for therapy are subjects of intense research efforts and both issues can be approached with the phage display technology.

1.1. Characterization of allergen epitopes

Molecular biology and biochemical techniques have considerably advanced the knowledge of allergens. Up to

now, descriptions of allergens have been based on recombinant DNA technology, chromatography, spectrometry of proteolytic fragments, crystallization, and X-ray investigations. These methods support the characterization of isoforms and the determination of the primary, secondary, and tertiary structures of these proteins. For further understanding of the pathogenesis of type I allergy, allergen epitopes for the humoral immune response should be known. Attempts to identify B cell epitopes of allergens on a molecular basis are limited by the fact that these epitopes are generally conformational and discontinuous in nature. The phage display technique is a relatively new tool to define peptide structures that mimic natural epitopes. Phage displayed random peptide libraries frequently yield peptides that mimic the binding site for an antibody but do not correspond to the linear sequence of the cognate antigen. These peptides are known as mimotopes and can represent linear and conformational epitopes as well as non-protein epitopes.

Employing this technique, our group was able to identify an IgG and an IgE epitope on the major birch pollen allergen, Bet v 1 [5,6]. By linear alignment, it was not possible to attribute mimotope sequences to the primary structure of Bet v 1. However, with the aid of a newly developed computer-aided coarse-grained epitope search, which compared amino acid properties and three-dimensional structure of the mimotopes and Bet v 1, it was possible to locate the two epitopes on distinct surface exposed areas of the molecule [6]. Similarly, an epitope on

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Der p 1, the major house dust mite allergen, was identified by utilizing the phage display technique [7].

Ultimately, the detailed analysis of discontinuous epitopes requires the resolution of the three-dimensional allergen structures by X-ray crystallography and/or nuclear magnetic resonance spectroscopy, with epitope mimics playing an important role in localizing the areas of interest on the molecule.

1.2. Epitope-specific immunotherapy

To date, the only causative treatment of type I allergies is specific immunotherapy (SIT). It is based on the administration of increasing doses of allergens to allergic patients, to yield allergen-specific non-responsiveness. SIT is a clinically proven effective treatment for allergic diseases, but despite having been in clinical practice since about a century, its use remains empirical. It could be demonstrated that successful immunotherapy is paralleled by an increase of IgG, in particular IgG4 antibodies [8]. Later studies, however, were unable to correlate the clinical improvement in immunotherapy of inhalant allergies with changes in the allergen-specific IgG and IgG-subclass level [9,10]. When allergen extracts are used for SIT, new IgE reactivities to previously non-IgE-binding allergen components are induced, which were observed by immunoblotting in 65% of the patients after one to three years of SIT [11]. The finding of immunotherapy-induced antibody responses against previously not recognized B cell epitopes indicates the vaccination character of this treatment [12]. The fact that patients start to mount *de novo* IgE as well as protective IgG responses against compounds of the extract may explain the unpredictability of SIT performed with allergen extracts. In fact, even the induction of food allergy during SIT has been shown [13], due to cross-reactivity of, e.g., profilins of pollen and fruit. SIT can also be associated with anaphylactic side-effects.

To overcome the problems inherent in the use of crude allergen extracts, recombinant DNA technology has been used to produce defined recombinant allergen molecules. A great advantage of recombinant allergen components is that they can be used for component-resolved diagnostics, which allow establishment of the patient's individual IgE reactivity profile and precise identification of the disease-eliciting molecules before selection of therapy. Component-resolved immunotherapy (CRIT), in due course, excludes the risk of therapy-induced sensitization against new allergens [14].

Today's SIT approaches should focus on the induction of IgG antibodies against IgE epitopes. These so-called "blocking antibodies," present in local secretions and serum, are directed to the same epitopes as specific IgE, but are not capable of binding to the high affinity IgE receptor on effector cells. Therefore, they will not only interfere with IgE–allergen interactions, but also

suppress allergen-dependent activation of IgE production. If blocking IgG antibodies interfere with IgE-mediated presentation of allergens to T cells, it may even be expected that T cell-dependent late reactions are also ameliorated.

Unfortunately, the induction of blocking antibodies requires high doses of allergens, which may result in anaphylactic reactions. Therefore, treatments should be performed with hypoallergenic recombinant or synthetic allergen derivatives with a low risk of anaphylactic side-effects. To reduce the allergenic activity of an allergen, it has to be changed in such a way that it cannot cross-link effector cell-bound IgE [15]. A multitude of approaches to obtain hypoallergenic allergen versions have been applied. Genetic engineering used point mutations to alter IgE binding profiles [16–19] and yielded, e.g., fragments that had lost their native fold [20] or allergen trimers that had lost their IgE binding capacity [21–23].

Another problem is that human IgG monoclonal antibodies raised against Bet v 1, the major birch pollen allergen, have been found to modulate the binding of specific IgE to the allergen in both directions: IgG antibodies may enhance or inhibit this interaction, depending on their own epitope specificity [24,25]. The enhancement seen was interpreted as IgG-induced stabilization of a conformational state in which certain IgE epitopes are better accessible [26] (reviewed in [27]). Thus, one needs to consider not only the component-resolved immunotherapy, but epitope-directed immunotherapy, to exclude the possible enhancement of allergic reactions. Continuous B cell epitopes were found by gene fragmentation, an approach applicable for allergens where the primary sequence is known. B cell epitope derived peptides, which lack secondary, tertiary structures and thus IgE binding capacity, were employed to induce and direct IgG to IgE domains (for allergens of which the IgE epitopes or three-dimensional structure is known) [28,29]. Another safe type of hypoallergenic fragments are such which only contain one IgE binding site (hapten principle) [30,31]. The application of the hapten concept for clinically practicable treatment of allergies requires that relevant cross-reactive allergens which harbor most of the relevant IgE epitopes are identified [32]. For tree and grass pollen allergens, it has indeed been shown by analysis of the DNA and deduced amino acid sequences, that unexpected structural similarities of many relevant allergens exist [33,34]. So a few recombinant allergens can resemble the relevant IgE epitopes of even complex allergen extracts. Data suggest that major allergens contain a few prominent B cell epitopes that may represent conserved immunodominant structures. In an analysis of three-dimensional structures of allergens, it was indicated that the relevant B cell epitopes mapped to surface exposed areas that are highly conserved across species and overlap with the binding sites of natural ligands [35].

IgE-binding haptens may be useful for active immunizations to focus IgG antibodies against IgE epitopes, thus ensuring blocking effects. Moreover, IgE-binding haptens could be used for passive therapy to saturate effector cell-bound IgE prior to allergen contact [32]. While haptens of continuous epitopes can be easily defined by fragmentation of the allergen-encoding DNA, haptens representing discontinuous IgE epitopes need to be rebuilt by more sophisticated technologies.

A very direct approach to obtain suitable IgE binding haptens is utilizing the phage display technology. As biopanning phage display libraries results in epitope mimics, there is no need for complicated remodeling of discontinuous epitopes. Allergen mimotopes generated with the phage display technology have been described for the plant panallergen profilin [36], the major birch pollen allergen Bet v 1 [5], the major house dust mite allergen Der p 1 [7], and for a cross-reactive grass pollen allergen [37]. When the peptides were displayed on phage, they were able to inhibit IgE binding to the natural allergen, which shows true mimicking properties and renders them interesting for vaccination trials [38]. In synthetic form, some of the mimotope peptides showed IgE inhibitory activity [37], whereas others did not [38]. For these, it was essential to define suitable carrier molecules that cause the peptide to adapt the same conformation as when displayed on phage [39]. Traditional carriers for peptides, as, e.g., keyhole limpet hemocyanin (KLH), can be applied for chemical coupling of mimotopes as well. However, it has to be considered that the mimotopes have to be coupled in the right orientation, i.e., coupling should be done via their C-terminus to the carrier molecule.

Mimotopes displayed on phage and on carrier proteins have been shown to elicit antibodies of desired specificities when used for immunizing mice. Indeed, vaccination with mimotopes of a monoclonal antibody known to enhance IgE binding to Bet v 1 resulted in antibodies again enhancing cross-linking and type I reactivity [40]. On the other hand, mimotopes selected with IgE induced IgG antibodies against the IgE epitope—which of course blocked subsequent IgE–epitope interactions [6]. Thus, mimotopes can be used to vaccinate against allergies in an epitope-specific fashion. They are safer than the current use of natural allergen extracts, which can evoke novel IgE responses, or recombinant allergens, which can still induce antibodies of undesired epitope specificities.

2. Description of materials and methods

2.1. Random peptide phage display libraries

Phage display libraries consist of filamentous phages particles displaying random peptides of defined length on

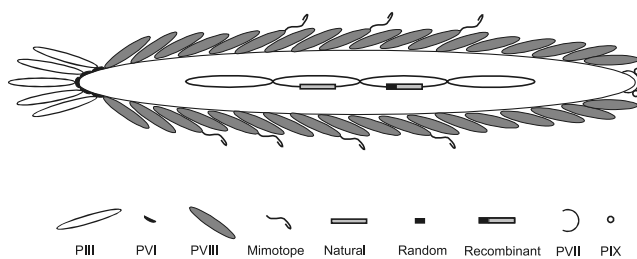


Fig. 1. Schematic drawing of a recombinant filamentous phage displaying fusion proteins on pVIII. The virion particle contains single-stranded DNA, coding for the various envelope proteins.

their surface. Filamentous phages particles have been genetically modified to carry foreign DNA sequences in their genome so that there is surface expression of the foreign protein fused to one of the phage coat proteins, either to minor coat protein pIII or—in a higher copy number—to major coat protein pVIII (Fig. 1). Each phage displays a unique foreign peptide, depending on the inserted gene [41]. Peptides may be of various lengths and libraries with 6, 7, 8, 9, 10, 12, 15, and 20 amino acids are described [42–46]. Peptides may be constrained by the insertion of cysteine residues that flank both sides of the insert, causing a cyclic peptide to be formed by disulfide bonds between the cysteine residues [47]. This may be advantageous and yield peptides with higher affinity for an antibody than unconstrained peptide libraries.

The mechanisms that have been developed for foreign gene expression by the phage may be loosely divided into two types, phage and phagemid. In the simpler phage system, the foreign cDNA is inserted directly into the phage genome, thus every copy of pIII or pVIII displays the foreign protein. If the inserted peptide disrupts the pIII or pVIII protein functionally, the phage will not be viable and the peptide will not be displayed. To meet this problem, phagemid systems were developed. The foreign cDNA is carried on a separate phagemid, that has only the phage gene, gIII or gVIII, into which the foreign sequence is to be inserted. To produce phage particles from this type of vector, a bacterium must also carry a helper phage with all the genes necessary for phage production, including a copy of the wild type gene used for display of the foreign protein in the phagemid. Therefore, both recombinant and wild type proteins will be produced and be incorporated into the phage particle. For pIII approximately one of the five copies and for pVIII from 1 to 30% of the 2700 copies will display the foreign peptide. The phage particle preferentially carries the phagemid DNA because the helper phage is so mutated that it replicates more slowly (reviewed in [48]).

2.2. Biopanning

Biopanning is the screening of phage display peptide libraries with antibodies (or any other binding

structures) of interest. From a phage display library comprising a repertoire of often far above 10^9 possible ligands, matching peptides are selected. Their surface characteristics are identical with those of the antibody's epitope, although their amino acid sequences may differ. By virtue of this mimicry, the mimotopes fit into the antibody's complementary determining region, independent of the original antigen sequence and composition. Thus, this technique obviates the need of knowing the three-dimensional structure or any post-translational adducts of target proteins.

Biopannings are performed according to the method of Parmely and Smith [49]. Several rounds of biopanning are done (usually three to four), starting with the original library, later using amplified phage eluents from the rounds before for a new selection (Fig. 2). For each round, 0.5–5 μ g of the desired antibody in 0.1 M NaHCO_3 (pH 8.5) is coated on ELISA plates (Nunc, Roskilde, Denmark) or polystyrene beads. After blocking with phosphate-buffered saline (PBS)/1% (w/v) dried milk at RT for 2 h, approximately 10^8 colony forming units (cfu) of phages (or 20 μ l of an original library) is incubated with coated antibodies at RT for 2 h. After extensive washing, bound phages are eluted. This can be done either by pH elution or by competitive elution. For *pH elution*, one can use glycine buffer at pH 2.2, incubate for 5 min, and neutralize the resulting eluent with Tris at pH 8.0. A recently described method to remove low-affinity phages and increase the recovery of high-affinity ones is to employ a stepwise reduction of pH in the final round of biopanning [50]. *Competitive elution* is possible when the structure that is to be mimicked is available in recombinant form. Then bound phages which truly mimic the binding site of the screening antibody can be displaced from the antibody by the natural ligand. This way, only specific mimics are obtained, in contrast to the pH method, where peptides with high affinity to the antibody, but only weak resemblance to the antigen

will also be eluted. Eluents are amplified by infecting freshly grown *Escherichia coli*. Bacteria are spun down and amplified phage particles in the supernatant are precipitated with polyethyleneglycol, spun down as well, and resuspended in Hepes (10 mmol/L)/NaCl (150 mmol/L). Amplificates are used for the following panning round. When all panning rounds are done, total phage titers of eluents are checked. An increase is a first indication of a successful outcome of the biopanning.

An increase of *specific* phage titers has to be checked consecutively by immunodotting or ELISA. For immunodotting, the selection antibody (and a control antibody) is dotted and incubated with the phage eluents from different biopanning rounds. For ELISA, the same principle applies, only that the specific antibody is coated in NaHCO_3 buffer overnight. Bound phages are detected with a labeled anti-phage antibody. The specific binding to the selecting antibody will ideally increase from round #1 to the last.

2.3. Colony screening

Colony screenings for selection of specific phage clones are performed after all panning rounds according to Barbas and Lerner [51]. In short, single colonies are grown and transferred to nitrocellulose membranes. After blocking, membranes are incubated overnight at 4°C with the selecting antibody (10 μ g/ml in PBS/0.15% (w/v) casein), an isotype control, or with buffer alone. Bound antibodies are detected with ^{125}I -labeled second antibody in PBS/0.15% (w/v) casein. Positive clones are selected according to signal intensities on autoradiographs.

2.4. Amplification and specificity control

Selected clones are amplified in *E. coli* and phage preparations are done with polyethyleneglycol as described above. These phages are double-checked in an ELISA again, as sometimes peptides adopt a different conformation on nitrocellulose than in solution. In this assay, the selection antibody is coated and incubated with phage clones as described above. Amplified wild type phage or the original library will be suitable controls.

2.5. DNA sequence analysis

Of positive single phage clones, DNA from overnight cultures is prepared using a standard M13 single strand phage DNS preparation kit. DNA sequencing is performed by the Sanger dideoxy method [52]. It may happen that the same sequence is found in an overwhelming number of clones. In that case, it should be considered that "overpanning" occurred. This means

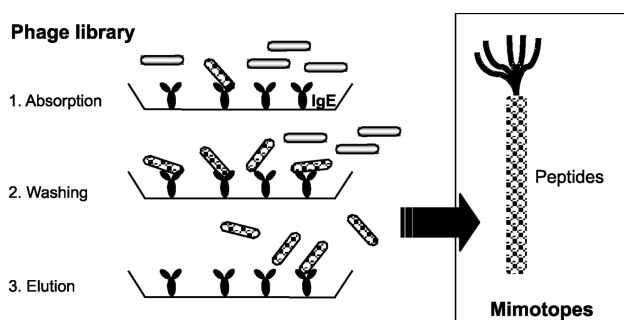


Fig. 2. A schematic biopanning round. Selection of specific phage clones from the repertoire of a phage display library. In a first step, phages bind to coated IgE, in a second step, non-binders are eliminated by stringent washing, and in a third step, specific phages are eluted, then further amplified, and used for the following panning rounds.

that clones with this insert have some growth advantage. For clarification, colony screenings of the previous panning rounds should be performed. It may, however, also indicate the selection of a clone with optimal binding characteristics to the complementary determining region of the selection antibody. Obtained sequences may be aligned with the original antigen's sequence, if this is known.

2.6. Mimicry testing and titration experiments

Per sequence obtained, one or two phage clones are tested for mimicry. In short, the selecting antibody is coated onto ELISA plates. Phages and original antigen are added in competition and bound phages are detected as described above. A control antigen should not be able to compete with phage-antibody binding. Another possibility is to coat the antigen (when it is available in purified form) and to preincubate the selecting antibody with the chosen phage clones. Detection is performed with an anti-selecting-antibody antibody. When mimicry is seen, the inhibition of these clones should be assessed further by stepwise reduction of phages or increase of antigen to estimate the relative affinities of peptide mimics. As the approximate copy number of fusion proteins is known (about 1 copy/phage in pIII display and 1000 copies/phage in pVIII for phagemid libraries, and five copies (pIII) or 2700 copies (pVIII) for phage libraries), a molar ratio of antibody/phage interaction may be calculated.

3. Concluding remarks

Several studies carried out so far have shown the usefulness of phage display in allergy research [5–7,36–40,53]. The possibility to obtain mimics of epitope regions of interest without the need of knowing the three-dimensional structure obviates complicated cloning, crystallizing, and modeling procedures, which have been necessary before. If this technique was applied more broadly, characterization of IgE epitopes on different allergens might be facilitated. For passive immunotherapy, mimotopes may act as haptens without cross-linking capabilities. For vaccine design, mimotopes have been shown to be an optimal approach, as they allow the induction of just the desired humoral immune response.

To generate potent immunizing preparations, still more research has to be dedicated to the question of carrier molecules. Phage-displayed mimotopes can be excellent immunogens when administered parenterally or orally [54,55]. They ensure the right conformation of the displayed peptide, however gavages of infectious particles are not feasible in humans for ethical reasons.

When produced synthetically, for most mimotopes there is a requirement that the peptides are constrained in order to retain their antigenicity. This could be as a result of their inherent flexibility when they are free in solution.

Another issue is that most mimotope peptides are too small to be immunogenic themselves. This problem is also solved by employing a suitable vehicle.

When working with mimotopes of the IgE epitopes of an allergen, the risk of cross-linking of cytophilic IgE in a sensitized individual should be considered. Obviously, spacing of the epitopes in an allergy vaccine is crucial, as giant allergen multimeres have been shown to be safe at least in type I skin testing [21–23]. Our group has previously presented a strategy using monovalent expression of IgE mimotopes with an antigenic carrier. From an immunological point of view, these monovalent mimotope constructs have been proven to be safe in animal experiments [39].

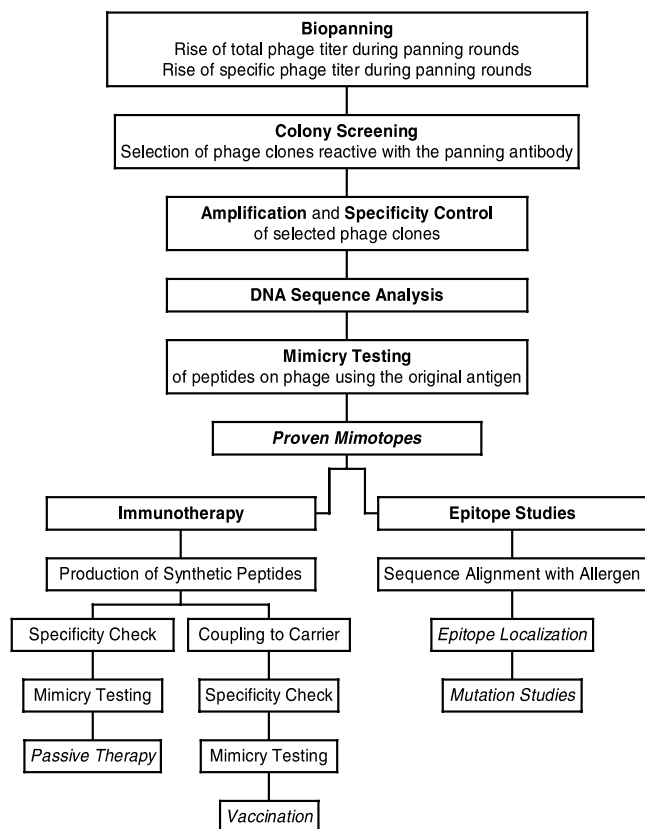
Taken together, synthetic subunit peptide vaccines are cost-effective and simple to produce, and are easily characterized during their manufacture. They are chemically stable and contain no infectious material. As active immunogens, they induce continuously available allergen-trapping antibodies. More importantly, they can focus immune responses to biologically relevant epitopes [56]. This capacity to narrowly focus the immune response is of particular relevance in allergy, where interaction of antibodies with specific sites of the allergen has the potential of enhancing IgE binding and, therefore, allergic symptoms.

Ultimately, carrier molecules are an essential requirement. When synthetic peptides are coupled to a carrier in the right orientation, one can even make a virtue of this necessity: mimotopes themselves are exclusive B cell epitopes, not able to attract any T cell help [53]. However, for eliciting high affinity antibodies, as preferred for blocking purposes, T cell help is crucial. A prudently chosen carrier will contain T helper cell epitopes and thereby recruit bystander T cells, to ensure isotype switch and affinity maturation. Another advantage of synthetic production of peptides is that they can also be formulated to include *several* T cell epitopes covering the MHC haplotypes for immunizing an out-bred population such as humans [56].

Displayed in a well-considered context, allergen mimotopes harbor great potential in allergy understanding and treatment.

3.1. Checklist for mimotope generation

Each step has to be successfully completed before commencing the next (Except for rises in phage titers during biopanning, which are only hints for success). Note: Some libraries may not be compatible with the antibody of interest.



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