

Peptide mimotopes as candidate vaccines

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The development of combinatorial peptide libraries, where random peptide sequences are displayed either on the surface of a phage or on a solid support, provides researchers with a powerful tool for analysis and study of the specificity of immune responses. The strength of this technology lies in the large amount of molecular diversity displayed that can be easily obtained and rapidly tested. As a result of screening peptide libraries, novel peptide sequences can be identified, which mimic native protective epitopes (mimotopes), and have the potential for use as vaccine candidates.

Keywords Mimotopes, peptide libraries, vaccines

Introduction

The disruption of the immune network by a foreign protein results in the induction of an immune response, which is predominantly focused at specific antigenic sites, known as epitopes. Since antibodies recognize B-cell epitopes that are mainly located on the surface of the molecule, the native conformation of the antigen is a critical parameter for this interaction to occur [1]. On the other hand, T-cells recognize linear peptide sequences (epitopes) presented by class I (cytotoxic T-cell epitopes) or class II (T-helper epitopes) major histocompatibility complex (MHC) molecules, generated after the processing of the antigen by antigen presenting cells (APCs) [2,3]. The identification of protective B- and T-cell epitopes can be advantageous for designing peptide vaccines against tumors, infectious diseases or allergic conditions, and is actively pursued in many laboratories around the world.

There are several advantages that make synthetic peptides an attractive option for vaccination: (i) they are chemically defined and easy to manufacture on a large-scale; (ii) they are stable and can be stored as a freeze-dried powder over prolonged periods; (iii) there is no infectious material involved in their manufacture and they are therefore safe; (iv) they elicit a predetermined type of immune response and undesired epitopes are avoided; (v) they can be designed to stimulate a broad immunity to cover different strains or serotypes of a virus; and finally (vi) they can be formulated to include several T-cell epitopes covering the MHC haplotypes for immunizing an outbred population such as humans. This article reviews recent applications of combinatorial peptide libraries to identify peptide mimics (mimotopes) of B-cell epitopes for potential use in vaccine development.

The development of the mimotope concept

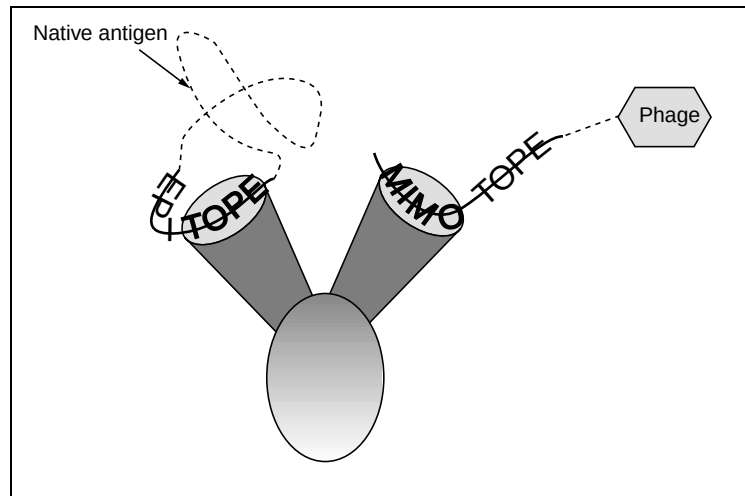
Since most antigens of biological importance are proteins, elucidating their antigenic profile is important for understanding the molecular mechanisms involved in immune recognition and selecting the appropriate epitopes for

vaccine development. Early studies on the antigenicity of globular proteins had suggested that antibodies recognize amino acids which either constitute a short linear stretch along the polypeptide chain (continuous or linear B-cell epitopes), or are brought together by the juxtaposition of the polypeptide chains when the protein is in its native conformation (discontinuous or conformational B-cell epitopes) [4]. Upon antigen entry into the immune system, a wide range of antigen-specific antibodies will be produced from the large pool of the available B-cell repertoire, with variable affinity constants. The majority of these antibodies recognize discontinuous B-cell epitopes, and there is only a small fraction of antibodies with specificity to continuous B-cell epitopes [5].

Considerable attention has been focused on the difficult problem of identifying the structural characteristics of B-cell epitopes. A number of empirical approaches have been employed to predict B-cell epitopes from the primary amino acid sequence of the protein, including the identification of regions of hydrophilicity, solvent accessibility, protrusion, atomic mobility and secondary structures (eg, β -turns). In spite of conceptual and practical difficulties, a small number of important protective B-cell epitopes have been identified. The predictive value of these methods is limited since they identify linear B-cell epitopes instead of discontinuous epitopes [4]. In addition, the inherent flexibility of short synthetic peptides in aqueous solutions is a major drawback in achieving the desired antibody specificity upon peptide immunization [6]. Anti-peptide antibodies normally bind with high affinity to the homologous peptide and only weakly to the native protein from which they are derived [7]. Several attempts to constrain peptide conformation (eg, by cyclization), had considerable success in improving the protective capacity of the induced anti-peptide antibodies [8], and increased the affinity of the interaction between the antigenic protein antibodies and the peptides [9]. Such constrained peptides presumably mimic the conformation of the cognate protective epitope on the native molecule.

Structural studies by X-ray crystallography of peptides or proteins complexed with monoclonal antibodies have greatly advanced our understanding of the nature of B-cell epitopes. This led to the realization that antigen recognition by antibodies is in fact at the level of atomic interactions, instead of recognition of a particular set of amino acid residues [4]. This suggests that structural features are critical for achieving the exquisite complementarity required between the paratope (binding site of immunoglobulin) and the B-cell epitope. It was Mario Geysen's pioneering work on the specificity of the antibody-antigen interaction which first introduced the term mimotope. It defines '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' (Figure 1) [10]. However, for a mimotope to be considered as a candidate for vaccine development both criteria of immunogenicity and antigenic specificity have to be fulfilled. That is to say, the mimotope should (i) mimic the antigenicity by binding to the antibody that recognizes the native epitope; and (ii) mimic the immunogenicity by eliciting antibodies cross-reactive with the native epitope that it mimics [11••].

Figure 1. Schematic presentation of an antibody recognizing an epitope from a native antigen and a mimotope displayed on a phage.



TOPE and MIMO are the parts of the epitope and the mimotope recognized by the antibody-binding site, respectively. MIMO mimics the structural characteristics of the TOPE.

Combinatorial peptide libraries

The development of combinatorial peptide libraries, where random peptide sequences serve as a pool of diverse 'molecular shapes' for selecting peptide ligands, provided a valuable tool for studying ligand receptor interactions, and more importantly for analyzing the specificity of immune responses [12•]. Such libraries can be constructed by cloning complex mixtures of combinatorially synthesized oligonucleotides into a display vector such as the filamentous phage (fd, f1 and M13), where the peptides (their length can range from 6 to 38 residues) are displayed as fusions at the N-terminal region of the phage coat proteins pIII or pVIII [13]. Because of the large number of peptides produced, it is necessary to test these peptides in pools rather than individually in order to identify the specificity of a given interaction. The surface display of peptides permits the selection of phage clones expressing sequences with high antigenic fitness to immobilized monoclonal antibody of unknown specificity. Phage displayed libraries presenting large peptides or cyclic peptides have the added advantage of being able to present a variety of secondary structures or constrained conformations respectively, which allows the selection of high affinity binders. Through a cycle of selection/rescue/amplification procedure (biopanning), clones of binding phages can be identified and finally sequenced to reveal the amino acid composition of the expressed mimotope.

Combinatorial peptide libraries can also be constructed by the solid-phase peptide synthesis of random sequences on a solid support (resin). By using the 'split synthesis' approach, Lam *et al* [14] have developed the 'one-bead one-peptide' methodology. Following interaction of a target molecule with the peptide bead, and using a simplified detection system based on ELISA, novel peptide sequences can be selected and identified by microsequencing. The use of phage-displayed peptide libraries offers several advantages over the use of

synthetic peptide libraries synthesized on beads: (i) sequence data can be obtained from a large number of phage; (ii) phages are easy to purify; (iii) the use of 'biopanning' allows for the selection of high affinity clones; and (iv) the phage can act as a carrier of selected sequences for the *in vivo* induction of antigen-specific antibody responses [15,16].

Screening peptide libraries to identify mimotopes of protective B-cell epitopes

B-cell epitopes and paratopes are relational entities that can be defined only by their mutual complementarity [4]. The molecular diversity displayed by a peptide library, allows the selection of ligands with structural complementarity to a given paratope. The first use of peptide libraries in immunology was for the identification of peptide sequences recognized by monoclonal antibodies [10]. Since these initial studies, a plethora of applications have been reported, and we will now describe some characteristic examples.

Peptide mimotopes of carbohydrate epitopes

Carbohydrate antigens normally induce a poor and short-lived antibody response in the immunized host. This is due to their inability to stimulate an MHC class II restricted T-cell response (T-independent antigens). As a consequence, carbohydrate antigens do not induce a sufficient secondary (anamnestic) response. The poor immunogenicity of carbohydrate antigens is particularly evident in young children who do not respond well to these antigens. Since carbohydrate antigens play a critical role in immunity, particularly against bacterial and fungal infections, and have shown promise in the immunotherapy of cancer, their conversion into T-dependent antigens could be advantageous for vaccine development. Replacing sugars with peptides identified by screening combinatorial peptide libraries might be a way to overcome their poor immunogenicity.

Carbohydrate antigens from group B streptococci (GBS), which are one of the major causes of neonatal sepsis and meningitis, are one example of microbial polysaccharides which are poor immunogens. Pincus *et al* [17] have screened a 9 mer phage-displayed peptide library against a protective monoclonal antibody specific for the type III capsular polysaccharide. Following selection of clones of binding phage by Western immunoblotting and sequencing of the displayed peptides, it was shown that within each of the two pools (comprised of ten clones each and selected on the basis of elution conditions), the sequence of each clone was identical. However, two very different sequences were revealed depending on the eluting pH, which probably reflects affinity differences between the two peptides. The specificity of the identified mimotopes was demonstrated by ELISA, where phage-displayed mimotopes were reactive not only with the parent monoclonal antibody, but also with polyclonal anti-GBS antibodies. In addition, the authors addressed the issue of structural resemblance between the identified peptide sequences and the carbohydrate epitope of GBS. In a series of inhibition studies they demonstrated that both the phage-displayed mimotope and the free mimotope could inhibit in a dose-dependent fashion the binding of the monoclonal antibody to the GBS-coated ELISA plates. Immunogenicity studies with the mimotope conjugated to a carrier protein have also shown that the identified peptide sequence could mimic the immunogenicity of the native carbohydrate epitope.

Studies with combinatorial peptide libraries have demonstrated that the aromatic motif W/YXY mimics several carbohydrate subunits. For example, the YPY motif is a mimic of mannose [18], while WLY mimics the Lewis Y carbohydrate antigen [19]. This raises the possibility that immunization with these peptides might result in the induction of antibodies that are cross-reactive with similar subunits expressed in dissimilar carbohydrate structures. The following two studies are good examples of the potential application of this concept for vaccine development. Agadjanyan *et al* [20] have recently examined the possibility of using mimics of the carbohydrate moieties of the envelope glycoprotein gp120 of human immunodeficiency viruses (HIV-1 and HIV-2) as immunogens in order to induce an antiviral neutralizing antibody response. The interest in these carbohydrate molecules as targets for vaccine development is based on the observation that: (i) the gp120 glycoprotein of HIV interacts with cells through carbohydrate molecules; and (ii) antibodies against them have been shown to neutralize field and laboratory isolates of HIV *in vitro*. By immunizing with a series of peptide mimics of mannose and lactoseries carbohydrate subunits, these authors were able to demonstrate that the anti-peptide antibodies bound to the glycosylated forms of HIV envelope protein more efficiently than the non-glycosylated form. In addition, the anti-peptide sera raised in two strains of mice displayed *in vitro* neutralizing activity against two HIV viral strains.

Based on the same principle described above, Kieber-Emmons and colleagues presented data on the ability of carbohydrate mimotopes to promote antitumor responses [21•]. In their study they examined the immunological mimicry of the W/YXY motif by designing peptide analogs with tandem repeats of this aromatic motif. Peptides were

synthesized as multiple antigen presentation systems (MAPs) and tested for immunogenicity in BALB/c mice. The peptides were shown to be immunogenic, inducing predominantly IgM antibodies which were reactive to neolactoseries antigens coated on ELISA plate, or expressed on human or murine tumor cells. More importantly, these antimimotope antibodies were shown to be functional since they could: (i) mediate complement-dependent cytotoxicity of human breast tumor cells expressing the Lewis Y neolactoseries antigen; and (ii) diminish tumor growth in mice immunized with mimotopes that were subsequently challenged with the sLeX-expressing Meth A sarcoma line.

There are several interesting points to note from these studies: (i) the use of combinatorial peptide libraries has changed our understanding of the concept of molecular mimicry. Antibodies do not necessarily bind to antigens of the same chemical composition or structure but also to chemically different antigens; (ii) peptide mimotopes identified by screening combinatorial peptide libraries can mimic not only the antigenicity of the native carbohydrate epitope but also its immunogenicity. These properties make them very good candidates for vaccine development; (iii) the sequence of the carbohydrate mimotopes contain several aromatic, acidic and hydrophobic residues. In particular, tryptophan and tyrosine residues appear to be the most prevalent.

However, it is still unclear whether the binding of these mimotopes to the parent antibody occurs by the same or different mechanism than the binding of the carbohydrate. The crystal structure of the complex of a *Cryptococcus neoformans* polysaccharide-specific monoclonal antibody with a peptide isolated from a phage-displayed library, proves that the mimotope binds to the antigen-binding site [22]. Furthermore, the antibody undergoes only minor conformational changes upon binding to the mimotope. Thus, for binding to occur the mimotope assumes the proper conformation to fit into the antibody-combining site. The crystal structures of Fab fragments with a single linear peptide have shown that in all cases the peptides do have some secondary structural elements, usually either a type I or type II β -turns, and it is the turn residues that are situated in the deepest part of the antibody-binding site [22]. In the particular case of the *Cryptococcus neoformans* mimotope, the β -turn containing tryptophan and methionine residues were shown to form an essential structural motif which allowed the mimotope to bind to the antibody-binding site [22].

Peptide mimotopes of protein epitopes

One of the advantages of screening random peptide libraries to identify B-cell epitopes is that there is no need to know the amino acid sequence of the protein against which the antibody had been elicited. Since peptide libraries provide a source of ligands (mimotopes) recognized by antibodies, the process of the identification of B-cell epitopes has been greatly simplified. In this section, we will describe characteristic examples of the screening of peptide libraries to map the specificity of monoclonal antibodies directed against proteins from pathogens or allergens, and to probe the specificity of the polyclonal antibody response in the course of a natural infection.

Allergen-specific mimotopes

With the current rise of incidence in allergic reactions, efforts to develop new strategies for immunotherapy have been intensified. One proposed approach is to block the binding of IgE, which is the dominant immunoglobulin in allergic reactions, to the allergen [23]. To this end, Jensen-Jarolim *et al* [24] used the phage display technology in order to define peptides that mimic conformational B-cell epitopes on the major birch pollen allergen Bet v1. Two random phage libraries (expressing linear or cyclic 9 mer peptides fused to the pVIII protein of filamentous phage) were screened against murine anti-Bet v1 monoclonal antibodies BIP1 and BIP4, which modulate the IgE binding capacity of the Bet v1 allergen. After screening with the BIP1 monoclonal antibody, the sequence CFPYCYPPSLR was found in seven out of ten analyzed clones. In contrast, when the BIP4 monoclonal antibody was used for screening, only one positive clone with the sequence CRQTRTMPGC was identified from 112 tested clones. Both natural and recombinant Bet v1 inhibited the interaction of the monoclonal antibodies BIP1 and BIP4 with phages expressing the corresponding peptides in ELISA, highlighting the true antigenic mimicry between the phage displayed mimotopes and the cognate epitopes. However, no inhibition was observed with the free peptides, suggesting that the mimotopes adopt the right conformation required for antigenicity only when presented as fusions to the pVIII protein on the phage. This was confirmed by NMR spectroscopy where the free peptides were shown to be conformationally heterogeneous in solution. The phage displayed BIP1-mimotope was also shown to be immunogenic after oral immunization, and induced serum IgG antibodies that cross-reacted with the Bet v 1 allergen when tested by Western immunoblotting.

Pathogen-specific mimotopes

In a recent study, Steward and colleagues have reported the characterization of one respiratory syncytial virus (RSV) neutralizing and protective monoclonal antibody (specific to the surface F glycoprotein of the virus), using a solid-phase library displaying random 8 mer peptide sequences on beads [25]. Following interaction of the library with the monoclonal antibody, five peptide sequences were selected which did not bear any sequence identity with the primary amino acid sequence of the F glycoprotein. In a series of experiments the authors addressed the importance of presentation of mimotopes for antigenicity. In particular, when the free mimotopes were tested for recognition by the monoclonal antibody in a peptide-based ELISA, no reactivity was observed and the peptides could not inhibit the binding of the monoclonal antibody to RSV in the fluid-phase. However, when the mimotopes were bound to resin beads or derivatized cellulose membrane, strong cross-reactivity with the monoclonal antibody was observed with mimotopes S1 (HWYISKPQ) and S2 (HWYDAEVL) and mimotope S1, respectively. It was then attempted to improve the antigenicity of the S1 mimotope by a series of substitutions, and several of them resulted in improved reactivity with the monoclonal antibody. Presentation of the mimotopes as multiple antigen peptides, resulted in highly antigenic and immunogenic constructs. Antimimotope antibodies were cross-reactive with RSV, neutralized the virus and inhibited the binding of the monoclonal antibody

to the virus. More importantly, following RSV challenge a 98.7% reduction of the virus load in the lungs was observed in mice immunized with one of these mimotopes.

The ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* is a protein that binds to the cytoskeletal structure of the erythrocyte after parasite invasion, and it has been suggested that it facilitates survival of the parasite *in vivo*. In a study by Adda *et al* [26] phage-displayed peptide libraries were used in order to characterize the antibody responses to the RESA protein. Following screening of the libraries with an immobilized monoclonal antibody, phage clones were isolated which displayed a number of mimotopes. Their sequences depended upon the type of phage library used and rounds of biopanning performed. When a 15 mer library was tested (expressing peptides as fusions to the minor coat protein pVIII, and present at five copies per phage particle), isolated peptide sequences were fairly diverse after two rounds of biopanning. However, following four rounds of biopanning, isolated sequences contained a common motif within different amino acid sequence contexts, as a result of the enrichment of phage with specific binding characteristics. Peptides isolated by screening a phage library displaying 250 copies of a 17 mer peptide (X15CX), with an invariant penultimate cysteine residue, as a fusion to the pVIII coat protein, were more diverse in sequence, and a common motif could not be identified. It is interesting to note that peptide sequences isolated from both libraries were significantly enriched by acidic residues but there was no identity with the RESA sequence. The binding specificity of the selected phage clones to the monoclonal antibody was confirmed by Western blot and ELISA, and by inhibition of binding of the monoclonal antibody to the selected phage by the RESA protein. Two synthesized free peptides were subsequently shown to inhibit the binding of the monoclonal antibody to the phage displaying the corresponding peptide, as well as most of the other phage-displayed mimotopes. Thus, it appears that the isolated peptides are mimicking structural features of the RESA. More interestingly, despite the sequence diversity of the mimotopes, all have the same fine specificity, suggesting that they are binding to subsites within the antigen-binding site [11••].

Although protective monoclonal antibodies are most commonly used for screening peptide libraries for B-cell epitopes, positive sera from patients have been tested as well. This enables: (i) the study of antibody specificities generated during the course of an infection; and (ii) the selection of the appropriate B-cell epitopes for vaccine development, particularly since some epitope specificities may change during the course of the disease as a result of virus mutation, antibody affinity maturation and/or viral escape. A good example of this approach is the work published by Scala *et al* [27•], who used libraries composed of random 9 mer peptides unconstrained or flanked by two cysteines, displayed on the N-terminus of the pVIII major coat protein of filamentous phages to screen HIV-1 positive sera. All selected clones (from both libraries) were reactive with positive sera from long-term non-progressor (LTNP) subjects and AIDS patients. It is interesting to note that serum samples manifested a distinct pattern of reactivity with the selected phage clones. Some sera recognized the majority of the phage clones indicating a broad antibody specificity,

whereas others were reactive with only one phage. Sequence characterization of the identified mimotopes has shown that some of the mimotopes shared homology with the primary amino acid sequence of gp120 and gp41 of various HIV isolates, whereas the remaining mimotopes did not. When mimotope-specific antibodies were isolated from positive sera by immunoaffinity purification, reactivity with HIV-1 proteins by ELISA was observed, confirming that the selected peptide sequences were true antigenic mimics of linear or conformational epitopes. These peptides were also shown to mimic the immunogenicity of the cognate epitope, since immunization of mice with the phage-displayed mimotopes resulted in the induction of antimimotope antibodies capable of neutralizing HIV-1 isolates.

All of these findings raise several interesting points:

- For some mimotopes there is a requirement that the peptides be constrained in order to retain their antigenicity [23,25,28]. This could be as a result of their inherent flexibility when they are free in solution, or because these mimotopes are low affinity binders.
- The antigenicity of the identified mimotopes can be further improved by selected substitutions.
- Identified mimotopes can either bear sequence homology with the antigen or no resemblance at all, suggesting that these novel peptide sequences are mimics of linear or conformational epitopes respectively. Since the binding site of an antibody is considered to be functionally polyspecific (it is not unique for a single antigen), several mimotopes with different amino acid sequences can be recognized by binding to different subsites within the binding site [11••].
- Screening random peptide libraries with positive sera has indicated that the antigenic polymorphism of a pathogen can be matched by a collection of epitopes selected for their affinity for pathogen-specific antibodies. These selected epitopes can be true antigenic and immunogenic mimics of the cognate epitopes.
- Phage-displayed mimotopes can be excellent immunogens when administered parenterally [15,16] or orally [24,29], inducing antibodies cross-reactive with the native antigen.

Conclusions

Screening random peptide libraries often results in the identification of novel sequences (mimotopes) which lack sequence similarity with the native antigen. These mimotopes can represent high affinity binders recognized by monoclonal or polyclonal antibodies to various antigens of different chemical nature. Moreover, they can mimic the immunogenicity of the cognate epitope and they are therefore potential candidates for vaccine development. The examples described are only a few of a wide range of possible applications of peptide libraries that are currently underway [30,31,32•]. Future efforts to improve the *in vivo* stability of mimotopes, for instance by using retroinverso peptides [33] may eventually lead their application in immunotherapy or vaccination becoming a reality.

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- of outstanding interest
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