Synthetic Peptide Vaccines and the Search for Neutralization B Cell Epitopes[#]

M.H.V. Van Regenmortel[*]

School of Biotechnology, University of Strasbourg, CNRS, Boulevard Sebastien Brand, 67400 ILLKIRCH, France

Abstract: Most attempts to develop synthetic peptide vaccines assume that it is possible to obtain effective vaccine immunogens by making short linear peptides adopt the structures observed when epitopes of pathogens are bound to neutralizing antibodies. Although more than a thousand synthetic peptides have been examined as potential prophylactic vaccines, only 125 peptides have progressed to phase I clinical trials, 30 have made it to phase II trials but not a single one has passed phase III trials and is currently marketed for human use.

Reasons for this lack of success include 1) an excessive reliance on continuous epitopes as vaccine candidates, 2) an exaggerated confidence in the specificity of antibodies, 3) the failure to recognize that an operational bias is introduced when monoclonal antibodies are used to characterize epitopes and, 4) a tendency to underestimate the difference between antigenicity and immunogenicity. There clearly is a need to overcome these misconceptions if synthetic peptide vaccines are ever to become a reality.

Key Words: Antigenicity, epitope prediction, immunogenicity, HIV, mimotopes, monoclonal antibodies, synthetic peptides, peptide-based vaccines.

INTRODUCTION

The B cell epitopes of proteins are the regions that are recognized by the binding sites or paratopes of antibody molecules, when these are present either in their free form in serum or as membrane-bound B cell receptors. They are called B cell epitopes to distinguish them from the T cell epitopes of proteins which are proteolytically cleaved peptides of the antigen that interact with the receptors of T cells. The present review will discuss only B cell epitopes and they will be referred to simply as epitopes.

In the context of vaccines, an important category of epitopes are the so-called neutralization B cell epitopes (sometimes mistakenly called neutralizing epitopes) that are able to elicit the formation of antibodies that neutralize the infectivity of pathogens. Such antibodies are known as neutralizing antibodies and the epitopes they bind to are mostly identified using neutralizing monoclonal antibodies (Mabs).

Attempts to develop synthetic peptide vaccines usually assume that it is possible to synthesize effective vaccine immunogens by making short linear peptides adopt the structures observed when epitopes of pathogens are bound to neutralizing antibodies. In theory, peptide-based vaccines could have many advantages compared to conventional vaccines such as increased safety and stability and lower cost [1]. More than a thousand synthetic peptides have been examined as potential prophylactic vaccines against viral, bacterial and parasitic infections [2-4] and as therapeutic vaccines for chronic infections and non-infectious diseases as well as cancer [5]. In a recent review [6], it was reported that although 125 peptides had progressed to phase I clinical trials and 30 peptides had undergone phase II trials, not one peptide vaccine had passed phase III trials and is currently marketed for human use. This striking lack of success in developing synthetic peptide-based vaccines suggests that some of the assumptions underlying these efforts were misguided. In the present review a number of misconceptions prevalent in this research area will be analysed. These include 1) an excessive reliance on so-called continuous epitopes as vaccine candidates, 2) an exaggerated confidence in the specificity of antibodies, 3) the failure to recognize that an operational bias is introduced when monoclonal antibodies are used to characterize epitopes and 4) a tendency to underestimate the difference between antigenicity and immunogenicity.

In view of the recent failure of the Merck HIV-1 vaccine trial aiming at cell-mediated immunity [7] it seems timely to review prospects for peptide vaccines that attempt to induce humoral immune responses.

THE NATURE OF PROTEIN EPITOPES

Epitopes of proteins are usually classified as continuous or discontinuous depending on whether the amino acids that constitute the epitope are contiguous in the peptide chain or not [8]. This terminology may lead one to believe that the units of recognition operative in antigen-antibody interactions are individual amino acids although it is at the level of individual atoms that interactions takes place. The vast majority of protein epitopes are discontinuous epitopes made up of atoms from residues located on two to five separate segments of the peptide chain brought together by the folding of the chain. These epitopes arise because the chain acts

*Address correspondence to this author at the School of Biotechnology, University of Strasbourg, CNRS, Boulevard Sebastien Brand, 67400 ILLKIRCH, France; E-mail: vanregen@esbs.u-strasbg.fr

[#]This review is the first one of a series of five review papers presented at the Vaccine Development Conference held at the University of Pittsburgh on 28-30 September 2008 (http://www.biacore.com/lifesciences/events/vaccine2008/home/index.html) which will be published in Open Vaccine Journal in the coming months.

1875-0354/09

as a scaffold to bring distant residues together and if the scaffold is perturbed, the epitope ceases to exist [9].

Fig. (**1**) shows the structure of a discontinuous epitope of the outer surface protein A (OspA) of the spirochete *Borrelia burgdorferi*, the etiological agent of Lyme disease. The structure of this epitope was established by X-ray crystallography of recombinant OspA complexed with the Fab fragment of mouse Mab 184.1 [10]. The OspA epitope consists of residues 30, 33-35, 42-46, 52, 69-71, 92-95 and 117-119 that are in contact with residues of the antibody paratope. When the epitope is represented as the disembodied set of residues identified by crystallography (Fig. **1B**) it becomes evident that this set of residues cannot be isolated as such from the OspA molecule to show that it possesses binding activity on its own. Discontinuous epitopes can only be identified by crystallography on the basis of atomic contacts with a paratope and not by showing experimentally that a set of non-contiguous residues in a protein, when positioned correctly in space (Fig. **1B**), possess binding activity.

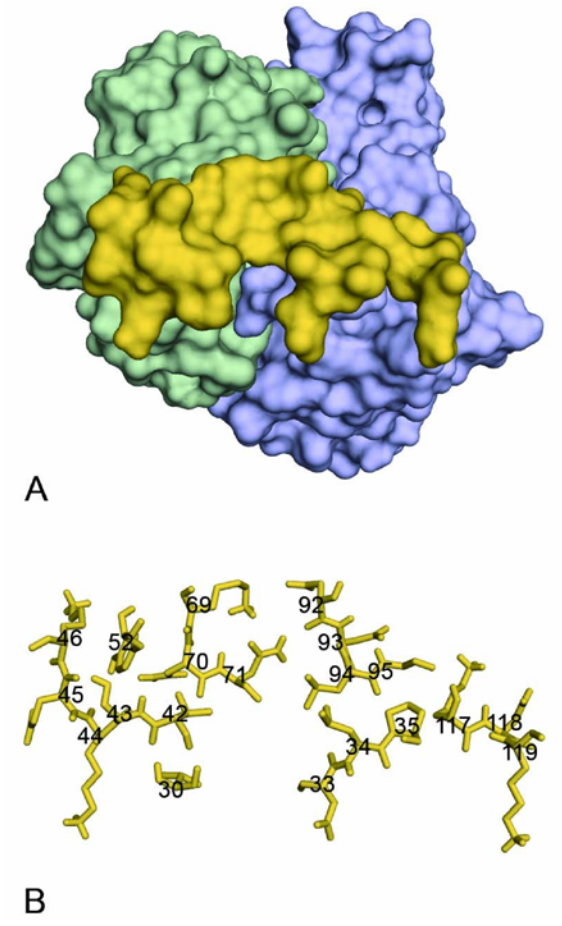

Fig. (1). Discontinuous epitope of the outer surface protein A of the spirochete *Borrelia burdorferi* elucidated by X-ray crystallography from a complex with Mab 184.1[ref 10]. (**A**). Outline of the epitope in yellow. (**B**). Position in space of the residues comprising the epitope. This set of residues cannot be isolated as such from the protein to show experimentally that it possesses binding activity on its own. Parts of the discontinuous epitope such as peptides 42-46 or 92-95 may be able on their own to bind to the antibody, in which case they would be called continuous epitopes of Osp A. (Courtesy Pernille Haste-Andersen, Danish Technical University).

The situation is completely different in the case of continuous epitopes of proteins since these epitopes are identified by showing experimentally that short linear peptide fragments of the protein are able to bind to antibodies raised against the protein. A continuous epitope is identified only by the binding activity of a peptide and not by showing that all the residues in this peptide interact with antiprotein antibodies. Usually, only some of the residues of a continuous epitope are part of an epitope present at the surface of the cognate native protein that is able to make contact with antibody molecules. In the case of the OspA protein, for instance, it is plausible that peptides corresponding to residues 40-48 and 90-98 (see Fig. **1B**) would be able to bind to Mab 184-1, since both these peptides contain a stretch of residues located at the surface of the protein. Such peptides could then be labeled continuous epitopes of the protein, although they contain only a few of the residues of the discontinuous epitope recognized by antibody Mab 184-1.

It is unfortunate that when one talks of the continuous epitopes of a protein, the impression is created that these epitopes exist as such in the native protein. In reality the label "continuous epitope" is given to any linear peptide that is able to react, usually only weakly, with antibodies directed to more complex discontinuous epitopes. Such peptides are not faithful copies of epitopes present in native protein molecules, one reason being that they do not retain the conformation present in the corresponding part of the folded protein; in most cases they possess only limited structural similarity with portions of the protein surface. It is not unusual for a short linear peptide such as a tripeptide or a pentapeptide to be called a continuous epitope, even if its binding activity is increased considerably when flanking residues are added to it. In the absence of structural information about which residues of the peptide are in contact with the antibody, is not clear if the longer peptides are more active because they possess a more appropriate conformation or because the added residues actually interact with the antibody [9]. As a result, continuous epitopes always have ill-defined boundaries since they are defined functionally rather than structurally. Functional epitopes are usually delineated by establishing which residue replacements in a protein or peptide affect its capacity to bind to a Mab. Such an approach not only identifies epitope residues that are in contact with the paratope but also residues that affect the epitope activity indirectly by altering the conformation of the peptide chain. Structural and functional approaches to epitope characterization therefore lead to different perceptions of the nature of epitopes [11].

Although large numbers of poorly characterized continuous epitopes are listed in many databases such as the Immune Epitope Database [12], the vast majority of them are very poor mimics of the actual epitopes present in native proteins. They become known as epitopes because the relationship between an epitope and its paratope is never of an exclusive nature and because antibodies are always able to cross-react with a wide variety of antigens that may possess only a limited degree of structural similarity [13]. Another reason for the large number of reported continuous epitopes is that many of them correspond to unfolded regions of denatured protein molecules that are not antigenically active in native proteins [14]. Antiprotein sera used for detecting continuous epitopes often contain antibodies specific for dena-

tured proteins because some of the protein molecules used for immunization became denatured before of after being injected in the animal [15]. Such antibodies usually do not react with the native protein but are able to bind to various linear fragments of the protein.

Since the vast majority of continuous epitopes reported in the literature do not correspond to the actual epitopes present in native proteins, it is not astonishing that continuous epitopes have not been successful as candidate peptide vaccines [6, 16]. An example of the problems encountered when attempts are made to turn a continuous epitope into a synthetic vaccine is provided by the heptapeptide ELDKWAS corresponding to residues 662-668 of the conserved membrane-proximal external region (MPER) of the gp41 protein of human immunodeficiency virus 1 (HIV-1). This peptide which reacts with the anti-HIV-1, broadly cross-reactive and neutralizing Mab 2F5, has been regarded as a promising vaccine candidate because it is located in a conserved region involved in the envelope-mediated fusion of the virus [17,18]. Various linear peptide constructs incorporating the ELDKWAS sequence have been synthesized using additional flanking residues and constraining the constructs in α-helical or β-turn like conformations [18-22]. Although some of the constructs had a higher affinity for the 2F5 antibody than the free, unconstrained heptapeptide, they were unable, when used as immunogens, to induce antibodies with detectable neutralizing capacity. The reason for this is that the ELDKWAS sequence actually corresponds to only a part of a larger and more complex discontinuous epitope that elicited the neutralizing Mab 2F5 [15, 18, 23, 24]. It seems that this region of the MPER is able to assume different conformations depending on the fusogenic state of gp41 and that its accessibility varies during the course of the infection process. There is also evidence that the viral membrane contributes to the structures recognized by several of the antibodies directed to the MPER region [18].

The inability of linear peptides to effectively mimic the discontinuous epitopes of proteins should come as no surprise since this has been the finding in hundreds of immunochemical analyses of protein antigens over the last two decades [25, 26]. It is unfortunate that so many investigators still rely on continuous epitopes for developing synthetic vaccines against pathogens since there is little experimental evidence that such a strategy is likely to be effective. In recent years, crystallographic evidence has also been obtained showing that short linear peptides cannot be effective structural mimics of discontinuous epitopes [27].

NEOTOPES AND MIMOTOPES

Two additional types of epitopes are important in the context of vaccines. Neotopes are epitopes specific for the quaternary structure of virus particles that arise only after the assembly of coat protein subunits into capsids. The term neotope was coined in 1966 [28] to describe epitopes that result either from the conformational changes in protein subunits induced by intersubunit interactions or from the juxtaposition of residues of neighbouring subunits that are recognized by an antibody as a single epitope [29]. For example, one neotope of poliovirus consists of residues 221-226 of VP1 protein together with residues 164-172 and 270 of VP2 protein [30]. Neotopes have been shown to be present in the capsids and membrane proteins of many viruses [31] and since the quaternary structure of virions can undergo major rearrangements following small changes in pH and temperature [32], neotopes are often transient epitopes [33] that can assume several conformations and are present for only short periods of time. The trimeric form of the envelope proteins of HIV-1 possesses transient neotopes, absent in the monomeric form, that are able to induce neutralizing antibodies [34].

The term mimotope was coined by Mario Geysen in 1986 [35] to refer to a peptide that is able to bind to a particular antibody but shows little or no sequence similarity with the protein antigen used to induce the antibody, usually because the antibody is directed to a discontinuous epitope. Mimotopes are mostly identified by testing combinatorial peptide libraries obtained by chemical synthesis or phage display and selecting peptides that bind to antiprotein antibodies. Even if the mimotope shows no sequence similarity whatsoever with the protein immunogen, it may sometimes be able to induce antibodies that cross-react with that protein [36]. The capacity of mimotopes to cross-react with antibodies to continuous and discontinuous epitopes of proteins demonstrates that epitope-paratope recognition does not occur at the level of whole amino acid residues but is mediated through individual atomic interactions that may occur through main chain atoms rather than side chain atoms of residues. The phenomenon of hydropathic complementarity also explains why peptide sequences that appear to have little in common are able to bind to the same antibody. Hydropathic complementarity arises from an inverted hydropathic pattern in two peptide sequences and is due to the attraction between hydrophilic and hydrophobic groups [37-39]. Peptide analogs that show little or no sequence similarity but retain the original hydropathic profile of the original peptide may be able to react with the same antibody. It was found, for instance, that an analog of a peptide fragment of angiotensin II was able to bind to short sequences from one hypervariable loop of an anti-angiotensin II antibody, provided the hydropathic profile of the angiotensin peptide analog was not altered by the residue substitution [40].

ANTIBODY MULTISPECIFICITY

The existence of mimotopes discussed in the previous section illustrates the ability of antibodies to react with a wide range of antigenic structures possessing little or no sequence similarity. The potential binding pocket of an immunoglobulin molecule comprises 50-70 hypervariable residues distributed over the six complementarity determining regions (CDR). However, an individual paratope consists mostly of only 10-20 CDR residues, which means that about two thirds of the CDR residues may be able to bind additional epitopes that bear little or no resemblance to a first bound epitope. A single immunoglobulin will thus always harbour a number of partly overlapping or nonoverlapping paratopes although, after binding to one epitope, it may not be able, because of steric hindrance, to accommodate a second epitope at a nearby location. This means that when an epitope is labelled a mimotope of epitope A because of its capacity either to bind to an anti-A antibody or to inhibit the binding of epitope A to the antibody, it cannot be excluded that the putative mimotope actually binds to a different paratope

from the one that interacts with epitope A. This is the reason why the mimotope nature of a peptide can only be established by showing that it is also able to elicit antibodies that cross-react with epitope A, i.e. with the epitope being mimicked [41-43].

The ability of antibodies to react with a large number of different epitopes that share only a limited degree of sequence or structural similarity may seem to contradict the accepted view that antigen-antibody interactions are very specific. In reality, antibody molecules are always able to react with many related antigens as evidenced by the large numbers of reported continuous epitopes and mimotopes.

Antibodies are often said to be specific for the particular antigen that was used for eliciting them in the immunized host. However, such a formulation can lead to considerable confusion. Proteins always harbour many different epitopes and each of them is able to elicit its own set of specific Mabs. When two viral strains are compared using a panel of Mabs, the strains will be indistinguishable if a Mab is used that recognizes an identical epitope that exists in both strains. On the other hand, if a Mab is used that recognizes an epitope present in only one of the strains, the two viruses will appear to be unrelated [44]. If an investigator wishes to differentiate between the two strains, he will call the first type of Mab non-specific since it reacts with both viruses while the second Mab will be called specific because it discriminates between the two strains. Instead of speaking of specificity, it is therefore preferable to speak of the discrimination potential of antibodies since this underlines the fact that it is the wish of the investigator to distinguish between two antigens that determines which antibody will be considered specific [44]. In fact, the paratopes of antibodies can only be said to be specific for their complementary epitopes and it makes little sense to say that an antibody is specific for a multi-epitopic antigen.

Another factor that contributes to the extensive cross-reactive potential of antibodies is the ability of the CDRs to adopt various conformations in their free states and when bound to different antigens. This flexibility of the CDR loops in fact increases the effective size of the antibody repertoire [45]. Furthermore, since all protein antigens exist as dynamic distributions of different conformers, this also increases the range of antibodies they can recognize and therefore also increases the number of cross-reactions that can occur [46, 47].

When the antigenicity of a protein is described in terms of the many peptides that are able to cross-react with antibodies raised against the protein, this does not imply that all the residues in these peptides correspond to residues of the protein immunogen that are able to react with antibodies. Peptide fragments and the corresponding regions in the intact protein usually differ considerably in conformation and the many analogs of continuous epitopes that are recognized by a single Mab further demonstrates the considerable multispecificity of antibodies [48].

Antibody specificity is often believed to be correlated with high affinity since it is expected that highly specific antibodies will possess a better stereochemical complementarity with their antigens than antibodies of low affinity. However, when the discrimination potential of antibodies is considered more relevant than their specificity, one often finds that antibodies of low affinity are able to discriminate better between two antigens than antibodies of high affinity. This is due to the fact that low affinity antibodies usually will detect fewer cross-reactions than antibodies of high affinity since weaker cross-reactions will tend more quickly to be below the level where they can be detected with low affinity antibodies [44].

THE OPERATIONAL BIAS OF MONOCLONAL ANTIBODIES

It is now widely accepted that the entire accessible surface of a protein antigen contains a very large number of overlapping epitopes [49]. The same residues at the protein surface can be part of neighbouring epitopes recognized by different antibodies and no sharp boundaries exist between these different epitopes which together form an antigenic continuum. The situation is somewhat analogous to the fuzzy boundaries between the colours in a rainbow which do not prevent us from distinguishing colours conceptually (Fig. **2**), in spite of the continuous nature of the spectrum of electromagnetic waves [44].

Fig. (2). A sculpture called “Rainbow Crash” by artist Federica Marangoni in the Chianti sculpture park, near Siena in Tuscany. Broken fragments of the rainbow appear as coloured pebbles which give the impression that there are clear boundaries between portions of the rainbow. In an analogous way, epitopes delineated with Mabs may hide the fact that the protein surface is an antigenic continuum. Epitope dissection with Mabs leads investigators to study immune responses elicited by single epitopes instead of analyzing the neutralizing activity observed in polyclonal immune responses.

It is only because antigenic sites of proteins are defined with Mabs that antigenicity appears to be located in discrete regions rather than in an antigenic continuum. Since in most cases only one or a very small number of Mabs have been used to characterize the epitopes of a protein by crystallography, it is difficult to assess if epitope regions differ from the remaining antigen surface that harbours additional uncharacterized epitopes [50]. In the case of lysozyme, five epitopes have been located by means of their respective Mabs and they were found to cover about two thirds of the lysozyme surface. When compared to the epitope areas, the non-epitopic surface was somewhat less accessible to solvent and contained fewer charged, polar and aromatic residues [50].

When the immune response to viral antigens is dissected with neutralizing Mabs, artificial boundaries are also created in what may be a functional continuum of several neighbouring neutralizing epitopes. The use of Mabs leads investigators to focus on single epitopes as elicitors of neutralizing antibodies instead of analyzing the protective immune response to a pathogen in terms of the collective neutralizing activities of antibodies directed to several epitopes. It is well-known that a normal polyclonal protective immune response is particularly effective because different neutralizing antibodies act in synergy and similar synergistic effects are observed when mixtures of neutralizing Mabs are used [51-54]. Different mechanisms have been suggested to explain the observed synergy between different neutralizing antibodies [55], one of them being that the binding of a first antibody to a viral protein induces conformational changes that expose new epitopes leading to enhanced binding by other antibodies. As a result, combinations of different neutralizing antibodies achieve a higher degree of neutralization than expected from the additive effect of each antibody taken individually.

Dissecting protective immune responses with Mabs leads investigators to concentrate on a single epitope that induces neutralizing antibodies instead of investigating which combinations of epitopes and immunogens would be most effective for eliciting a synergistic protective effect. In this respect, it is unfortunate that few investigators have analyzed in detail the immune response induced by whole HIV particles inactivated by heat treatment or various chemical treatments [56, 57]. Studies using inactivated viruses or virus-like particles [58] endowed with an increased expression of oligomeric envelope proteins in a particular conformation could help to unravel which combinations of immunogenic epitopes are able to mediate a more potent neutralization than is obtained with purified recombinant envelope proteins or single peptide epitopes [58].

The overlapping nature of the epitopes recognized by two anti-lysozyme Mabs elucidated by crystallography is illustrated in Fig. (**3**) [59]. The two antibodies showed no sequence similarity in their CDRs and the orientation of the two sets of hypervariable loops with respect to the antigen surface was completely different. Thirteen lysozyme residues were recognized by both Mab F9-13.7 (Fig. **3A**) and Mab HyHEL10 (Fig. **3B**) but the bonding patterns between epitope and paratope in the two complexes were very different.

Mab HyHEL10 formed a salt bridge between lysozyme residue K97 and residue D32 of the H1 antibody loop (Fig. **3B**) while Mab F9-13.7 formed salt bridges between the three lysozyme residues , K97, K96 and H15 and respectively residues E50, D52 and D54 of the H2 loop (Fig. **3A**).

Other differences between the two epitopes were that residues T89 and G102 contributed only to the epitope recognized by Mab HyHEL10 (Fig. **3B**) whereas residue N77 was only part of the epitope recognized by Mab F9-13.7 (Fig. **3A**). Although there is considerable overlap between the two epitopes regarding the residues that are in contact with two very different paratopes, the different bonding patterns exhibited in the two complexes show that we are dealing with two different epitopes. This example shows that the same residues of a protein can be involved in different epitopes, each one recognized by a separate Mab. Although it is not possible to draw a sharp distinction between what are very similar but not identical epitopes, the relational nature of epitopes and paratopes implies that as soon as an epitope has been altered slightly and binding to the antibody is affected, both the epitope and the paratope are no longer the same.

The epitope nature of a set of amino acids can only be revealed when an immunoglobulin that binds to it has been found. Similarly, the antibody nature of an immunoglobulin becomes apparent only when a complementary epitope to its paratopes has been identified. Epitopes and paratopes are relational entities defined by their mutual complementarity and they depend on each other to acquire a recognizable identity [9, 44]. An epitope is thus not an intrinsic structural feature of a protein that could be recognized in the absence of a particular interaction with a paratope. Epitopes acquire their identity by virtue of a relational nexus with complementary paratopes and this relational dependence means that analyzing the antigenicity of a protein amounts to analyzing the size of the immunological repertoire of the host immunized with that protein. The number of epitopes present in a protein can be equated with the number of different Mabs that can be raised against it. Using that criterion, the insulin molecule was found to possess at least 115 different epitopes [60] while the BLyS molecule possesses as many as a thousand epitopes [61].

Another bias introduced when Mabs are used to characterize protein epitopes is that the specificity of the antibody is determined by the selection process which was used to obtain the Mab. For instance, if a Mab has been selected using a library of linear peptides hypothesized to mimic parts of a continuous epitope of a virus protein, it would be somewhat of a self-fulfilling prophecy to discover subsequently that such an antibody binds more strongly to peptides than to intact virus particles.

It seems that many investigators do not always fully appreciate the bias that is introduced by the selection process used to obtain Mabs, since in many publications describing the binding properties of Mabs, no mention is made of the antigen that was actually used in the selection process. In the case of human Mabs 2F5, 4E10 and Z13 that recognize the gp41 MPER of HIV-1, various peptides were used for selecting the antibodies and some short peptides became known as

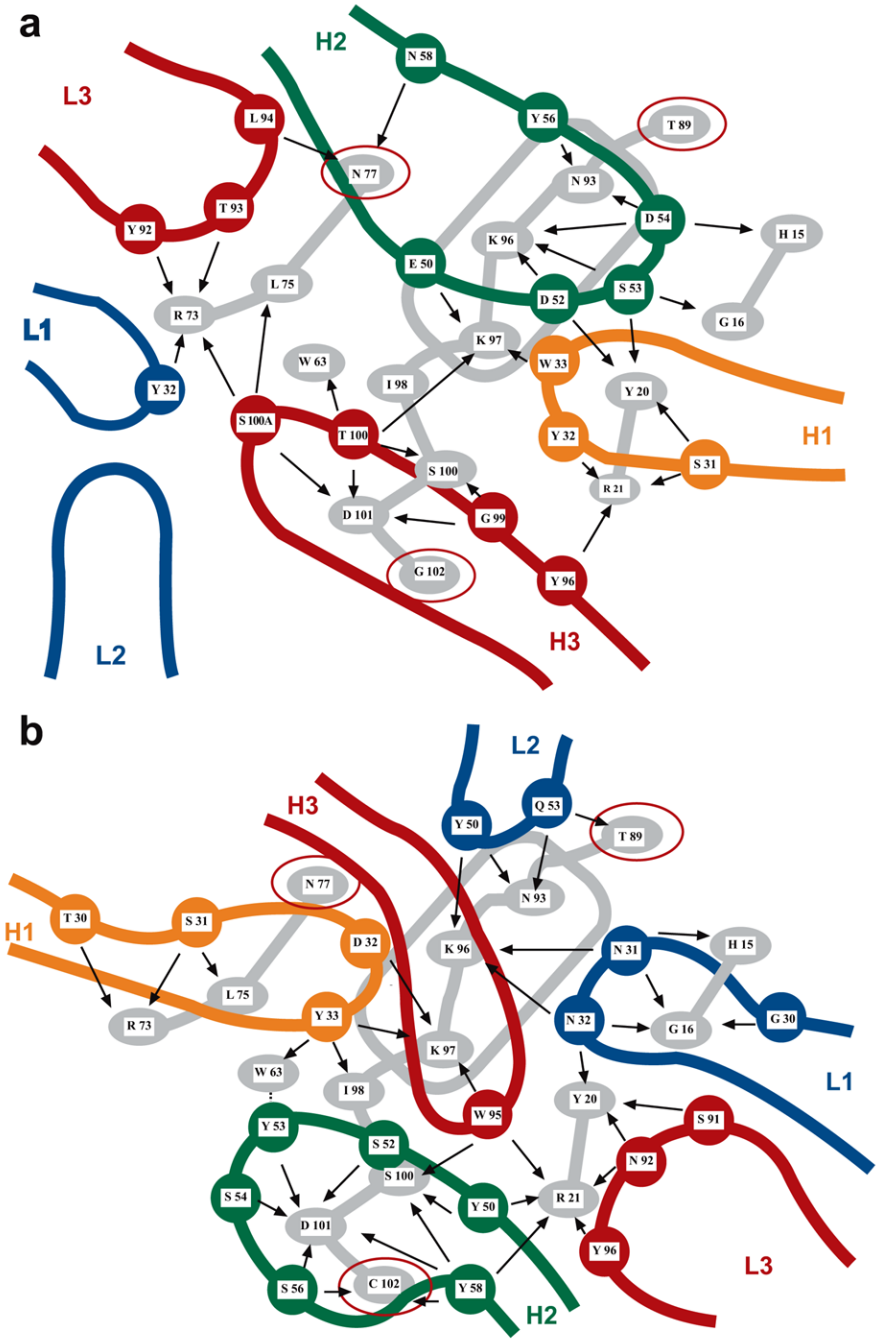

Fig. (3). Two overlapping discontinuous epitopes of lysozyme recognized by Mabs F9-13.7 (**A**) and HyHEL 10 (**B**) elucidated by X-ray crystallography. Thirteen residues of lysozyme (in gray) are recognized by both antibodies, albeit with different bonding patterns. The rounded rectangle in gray represents the lysozyme α-helix. The CDRs are shown in colour. Three residues (N77, T89 and G102 highlighted with red circles) are not shared by the two epitopes. Intermolecular contacts are shown by arrows. (adapted from Lescar *et al.*, J Biol Chem, 1995; 270: 18067 and reproduced with permission).

the core epitopes recognized by these Mabs [33]. Considerable work was then undertaken to try to convert these peptides into immunogens capable of eliciting antibodies with the same neutralizing capacity as the original Mabs [18, 21, 62, 63]. As discussed above, these misguided attempts failed because these so-called epitopes were not recognized for what they actually were: short peptides corresponding to small parts of complex discontinuous epitopes.

Another interesting case are the human Mabs directed to the V3 variable region of the gp120 envelope protein of HIV-1. The V3 loop which has been called the principal neutralizing domain of the virus consists of about 35 amino acid

residues and is able to induce the formation of antibodies that have a potent neutralizing activity against T-cell line-adapted (TCLA) strains of HIV [64, 65]. By absorbing human anti HIV-1 sera with linear V3 peptides, it was possible to remove the antibodies that neutralized TCLA strains but not those that neutralized primary HIV-1 isolates less well [66-68]. This led to the erroneous conclusion that the antibodies present in human antisera which neutralized the primary isolates were not directed against the V3 loop. This misinterpretation was due to the failure to recognize that these neutralizing antibodies were recognizing a specific V3 conformation. When human anti-V3 Mabs were selected with a V3 fusion protein (V3-FP) which retained the V3 loop conformation present in the virus [69], the antibodies were found to react more strongly with conformationally intact V3 than with linear V3 peptides. These Mabs which neutralized TCLA strains also possessed significant neutralizing activity against primary isolates from several HIV-1 clades [70] and recognized a V3 conformation present in the virus, in V3-FP and in a disulfide-bonded V3 loop but not in linear V3 peptides. This explains why in earlier experiments, absorption of anti-HIV-1 human sera with linear V3 peptides failed to remove antibodies that neutralized primary HIV-1 isolates. For the same reason selecting Mabs with linear V3 peptides also did not yield antibodies capable of neutralizing primary HIV-1 strains [71].

THE CROSS-PROTECTIVE IMMUNOGENICITY OF PEPTIDES CANNOT BE PREDICTED FROM THEIR ANTIGENICITY

Many investigators do not fully appreciate the considerable difference between the antigenicity and immunogenicity of peptides and proteins. The antigenicity of peptides is a purely chemical property describing the interactions between epitopes and paratopes in terms of structural and chemical complementarity. In contrast, the immunogenicity of peptides is their ability to give rise to an immune response which is a biological property that has meaning only in the context of a competent host. It depends on extrinsic factors such as the host immunoglobulin repertoire, the presence of appropriate B cell receptors, self tolerance, the production of chemokines and various cellular and regulatory mechanisms existing only in the biological context of an immune system [41].

When a protein epitope recognizes a free antibody molecule or a B cell receptor embedded in a membrane, the chemical environment is not the same [17] which means that the antigenic epitope bound to a free antibody molecule may not be identical to the immunogenic epitope that interacts with a B cell receptor.

It is commonly found that when a peptide fragment of a protein is able to bind to antibodies raised against the protein, this does not guarantee that the peptide will necessarily be able to elicit antibodies that react with the native protein. When the peptide reacts in an immunoassay with an antibody directed to a native protein, the antibody may be able to select one conformation of the peptide or it may induce a reactive conformation in the peptide by an induced fit or mutual adaptation process, the result in both cases being the occurrence of a cross-reaction between the peptide and the antiprotein antibody. In contrast, during the immunization process, when the same peptide is confronted with a variety of B cell receptors, different conformations of the peptide may be recognized by separate B cell receptors. However, there is no reason why the peptide would bind preferentially to those rare receptors which in addition to recognizing one conformation of the peptide also cross-react with a related epitope present in the native protein. It is thus to be expected that most elicited antipeptide antibodies will not react with the native, cognate protein, unless the peptide used for immunization was constrained and mimicked exactly the conformation of the corresponding region in the native protein immunogen.

Attempts to determine the conformation of epitopes present in immunogens are mostly based on X-ray crystallography or NMR studies of antigen-antibody complexes. However this approach is bedevilled by the fact that the structures visualized in the complexes may be different from the structures of the binding sites in free antigen and antibody molecules before the process of mutual adaptation that occurs when the two partners interact [72-78]. In spite of this limitation, many groups continue to use this approach and are not deterred by the fact that structural data obtained from complexes with neutralizing Mabs will not necessarily reveal the epitope structure recognized by B cell receptors during the immunization process and which is therefore likely to be required for eliciting neutralizing antibodies. Since it is known that all antibodies including Mabs are multispecific, it follows that the epitope structure observed in a complex is only one of the many antigenic binding sites that could be accommodated by the antibody. The epitope structure observed in a complex with a neutralizing Mab is thus not necessarily the one that corresponds to the immunogenic epitope which the investigator is trying to elucidate.

The type of immunogenicity required for a peptide to be considered a valid synthetic vaccine candidate can be defined as follows. Most peptides are immunogenic in the sense that they readily elicit antibodies that react with the peptide immunogen [79]. However, this type of immunogenicity is irrelevant for vaccination purposes since what is required is both cross-reactive and cross-protective immunogenicity, i.e. the induction of antibodies that recognize the parent protein and neutralize the infectivity of the pathogen harbouring the antigen [15].

Cross-reactive immunogenicity can often be obtained by increasing the conformational similarity between peptide and intact protein for instance by cyclization of the peptide but such an approach is rarely successful for achieving cross-protective immunogenicity [16]. It is sometimes possible to obtain a more suitable epitope conformation by inserting a peptide at certain locations in a recombinant protein [80-82]. However, in the absence of information regarding which precise conformation is required and is actually present in the recombinant construct, such a strategy remains entirely empirical.

The difficulties encountered when one tries to utilize a suitable peptide conformation for eliciting the formation of neutralizing antibodies are illustrated by the V3 peptide of HIV-1. This semiconserved and rather flexible loop of about 35 residues, which determines which co-receptor (CCR5 or CXCR4) is used by the virus to gain entry into

cells, is recognized by many HIV-1 neutralizing antibodies present in infected inviduals. It is known that the V3 loop is able to induce anti HIV-1 neutralizing antibodies [33, 83, 84] but the precise V3 conformation that should be present in a V3 synthetic peptide vaccine has not been established. NMR studies of linear peptides complexed with Mabs specific for the conformation of the V3 loop present in intact virus and in gp120 protein showed that the conformation of the antibody-bound V3 peptides was dictated by a process of induced fit to each Mab [85]. Alternative β hairpin conformations could be induced in the same V3 peptide depending on whether it binds to a Mab neutralizing a broad spectrum of virus isolates or to a Mab able to neutralize only a single type CXCR4 virus. Such findings once again demonstrate that the epitope conformation observed in Mab-peptide complexes is actually induced by the binding process and that structural analysis of complexes does not necessarily indicate which immunogen conformation is required for eliciting neutralizing antibodies.

EPITOPE PREDICTION AND THE SYNTHETIC RECONSTRUCTION OF DISCONTINUOUS EPITOPES

The main objective of epitope prediction is to be able to replace a complete protein antigen by a small fragment of the molecule corresponding to a single epitope. Ideally such a fragment can subsequently be synthesized as a peptide and used either to detect specific antibodies for immunodiagnostic purposes or to elicit antipeptide antibodies for antigen detection or as a potential synthetic vaccine [26, 86, 87]. Most attempts to predict epitopes have analyzed protein sequences in an effort to identify short surface-exposed regions of 5-10 residues that are particularly hydrophilic and accessible to the solvent and therefore likely to be recognized by antibodies [88, 89]. Since sequence-based prediction methods will only identify continuous epitopes that are known to be poor mimics of the epitopes present in native proteins, it is not astonishing that the success rate of these prediction methods rarely exceeds 60% correct predictions [90-92]. Furthermore the relevance of predicting such epitopes in the context of vaccine development is doubtful, since as discussed earlier in this review, relying on continuous epitopes for developing synthetic vaccines has not been an effective strategy.

It has been claimed [93] that epitope prediction methods based on the analysis of protein sequences using amino acid propensity scales also allows the prediction of discontinuous epitopes. However, if the 3D protein structure is unknown, such an approach only provides a list of contiguous and non-contiguous residues that are likely to be accessible at the protein surface, without an indication of how these residues must be positioned in space to make up a discontinuous epitope [94]. If prediction of discontinuous epitopes is limited to predicting that certain surface residues are likely to be part of an epitope, this does not amount to predicting the epitope itself since it is necessary to predict which residues from distant parts of the sequence must be brought together in a precise configuration to form an antigenically active site. Since predicting an epitope means predicting something that has a functional activity, many of the published methods for predicting discontinuous epitopes are actually misnomers.

Several prediction methods that take into account the 3D structures of antigens have been published [89]. The CEP (Conformational Epitope Prediction) web server predicts discontinuous epitopes by collapsing predicted continuous epitopes for which the Cα atoms are within a distance of 6 Å [95]. The DiscoTope method uses a combination of hydrophilicity parameters, amino acid statistics, numbers of contacts and area of relative solvent accessibility [96]. The MIMOP computational prediction tool [97] identifies key residues from sets of mimotope sequences and matches them with accessible amino acids on the antigen surface. The PEPOP tool [98] uses the 3D coordinates of proteins to predict clusters of surface accessible segments of the peptide chain. The Mapitope algorithm [99] also uses information from sets of mimotopes to identify key residue pairs and then maps these on the known surface of the antigen. The PEPITO predictor uses a combination of amino acid propensity scores and half sphere exposure values at multiple distances and achieves "area under the curve" prediction values of 68.3% [100]. All these methods succeed in predicting a number of residues that are part of known discontinuous epitopes but it remains to be seen whether they can provide information useful for the development of peptide vaccines.

Instead of relying on epitope prediction methods to infer which synthetic peptides are likely to be the best candidates for developing a vaccine, some investigators have used a more empirical approach and synthesized peptides that were constrained in a particular conformation in an effort to make them resemble the conformation of the corresponding region in the parent protein [6, 79]. The ability of such peptides to induce antibodies cross-reactive with the cognate protein and endowed with neutralizing activity can then be determined experimentally. Attempts to reconstitute discontinuous epitopes in this manner have so far only been moderately successful [101-105] although it is undeniable that such experiments provide highly relevant information on the vaccination potential of synthetic peptides that mimic complex discontinuous epitopes. A particularly promising approach is the CLIPS-technology (Chemical Linkage of Peptides onto Scaffolds) which combines the chemical linkage of a linear peptide to a synthetic scaffold with conformational fixation of the peptide [106, 107]. This method can be used for constraining the conformation of free peptides in solution as well as for obtaining solid phase-attached peptides in microarrays useful for mapping epitopes recognized by neutralizing antibodies. A strategy such as the CLIPS-technology is likely to be more effective than approaches that attempt to reconstitute so-called epitope hot spots. Hot spots in binding interfaces are regions that contribute most to the interaction binding energy, since when hot spot residues are mutated, the binding constant tends to be decreased about 100-fold [108, 109]. However, residues identified in this way do not represent the full immunogenic site required for eliciting neutralizing antibodies and reconstructing hot spots by synthesis is unlikely to lead to effective vaccines.

CONCLUSION

The search for neutralization epitopes that could be used to develop synthetic peptide vaccines has so far met with little success. Some of the misconceptions discussed in this review together with the dynamic nature of epitopes are

probably responsible for this state of affairs although another factor is the common assumption that it should be possible to develop successful peptide vaccines by structure-based rational design [24, 109, 110]. It is unfortunately the case that we still do not understand why some epitopes are able to elicit neutralizing antibodies while others are not and this prevents us from producing neutralization epitopes by design. This means that only an empirical, trial and error, approach may eventually succeed in identifying which synthetic immunogens are capable of protecting against disease. Immunizing animals with a variety of well-chosen immunogenic constructs and analyzing the resulting immune responses with respect to neutralizing capacity may well be the strategy that is most likely to succeed for developing an HIV vaccine. It is sometimes claimed that the animal experiments needed for studying immunogenicity are unacceptably expensive. However, such experiments may in fact be less costly than some of the existing and well funded programs that attempt to elucidate the 3D structures of hundreds of antigen-antibody complexes and it is conceivable that they might provide information that will be highly relevant for vaccine development. In view of our inadequate understanding of structure-function relationships in neutralizing antibody molecules [25, 109, 110], the current emphasis on structural analysis of vaccine antigens should be counterbalanced and complemented by more extensive studies of the functional activity of candidate vaccine immunogens and the antibodies they elicit.

ACKNOWLEDGMENTS

The author is grateful to Pernille Haste Andersen of the Danish Technical University, Denmark, for providing Figure 1 and to Florence Diemer for excellent secretarial assistance.

ABBREVIATIONS

BlyS	=	B-lymphocyte stimulator protein
CDR	=	Complementarity determining region of an antibody
HIV-1	=	Human immunodeficiency virus 1
Mab	=	Monoclonal antibody
MPER	=	Membrane-proximal external region of gp41 of HIV-1
NMR	=	Nuclear magnetic resonance
OspA	=	Outer surface protein A of *Borrelia burgdorferi*
TCLA	=	T cell line adapted (HIV strain)
V3-FP	=	V3 peptide fusion protein

REFERENCES

[1] Van Regenmortel, M.H.V.; Muller, S. In *Synthetic Peptides as Antigens*; Van Regenmortel, M.H.V.; Muller, S., Eds.; Elsevier: Amsterdam, **1999**, pp. 281-317.

[2] Arnon, R. *Synthetic Vaccines*; CRC Press: Boca Raton, FL, **1987**.

[3] Nicholson, B.H. *Synthetic Vaccines*; Blackwell Scientific Publishers: Oxford, **1994**.

[4] Arnon, R.; Ben Yedidia, T. Old and new vaccine approaches. *Int. Immunopharmacol.*, **2003**, *3*(8), 1195-1204.

[5] Sundaram, R.; Dakappagari, N.K.; Kaumaya, P. Synthetic peptides as cancer vaccines. *Biopolymers*, **2002**, *66*(3), 200-216.

[6] Hans, D.; Young, P.R.; Fairlie, D.P. Current status of short synthetic peptides as vaccines. *Med. Chem.*, **2006**, *2*(6), 627-646.

[7] Watkins, D.I.; Burton, D.R.; Kallas, E.G.; Moore, J.P.; Koff, W.C. Nonhuman primate models and the failure of the Merck HIV-1 vaccine in humans. *Nat. Med.*, **2008**, *14*(6), 617-621.

[8] Atassi, M.Z.; Smith, J.A. A proposal for the nomenclature of antigenic sites in peptides and proteins. *Immunochemistry*, **1978**, *15*(8), 609-610.

[9] Van Regenmortel, M.H.V. In *Synthetic Peptides as Antigens*; Van Regenmortel, M.H.V.; Muller, S., Eds.; Elsevier: Amsterdam, **1999**, pp. 1-78.

[10] Li, H.; Dunn, J.J.; Luft, B.J.; Lawson, C.L. Crystal structure of Lyme disease antigen outer surface protein A complexed with an Fab. *Proc. Natl. Acad. Sci. USA*, **1997**, *94*(8), 3584-3589.

[11] Van Regenmortel, M.H.V. Structural and functional approaches to the study of protein antigenicity. *Immunol. Today*, **1989**, *10*(8), 266-272.

[12] Zhang, Q.; Wang, P.; Kim, Y.; Haste-Andersen, P.; Beaver, J.; Bourne, P.E.; Bui, H-H.; Buus, S.; Frankild, S.; Greenbaum, J.; Lund, O.; Lundegaard, C.; Nielsen, M.; Ponomarenko, J.; Sette, A.; Zhu, Z.; Peters, B. Immune epitope database analysis resource (IEDB-AR). *Nucleic Acids Res.*, **2008**, *36*, (web server issue) w513-w518.

[13] Roberts, V.A.; Getzoff, E.D.; Tainer, J.A. In *Structure of Antigens*; Van Regenmortel, M.H.V., Ed.; CRC Press: Boca Raton, FL, **1993**, Vol. *2*, pp. 31-53.

[14] Laver, W.G.; Air, G.M.; Webster, R.G.; Smith-Gill, S.J. Epitopes on protein antigens: misconceptions and realities. *Cell*, **1990**, *61*(4), 553-556.

[15] Van Regenmortel, M.H.V. Immunoinformatics may lead to a reappraisal of the nature of the B cell epitopes and of the feasibility of synthetic peptide vaccines. *J. Mol. Recognit.*, **2006**, *19*(3), 183-187.

[16] Van Regenmortel, M.H.V. Molecular design versus empirical discovery in peptide-based vaccines. Coming to terms with fuzzy recognition sites and ill-defined structure-function relationships in immunology. *Vaccine*, **1999**, *18*, 216-221.

[17] Zwick, M.B. The membrane-proximal external region of HIV-1 gp41: a vaccine target worth exploring. *AIDS*, **2005**, *19*(16), 1725-1737.

[18] Montero, M.; van Houten, N.E.; Wang, X.; Scott, J.K. The membrane-proximal external region of the human immunodeficiency virus Type 1 envelope: dominant site of antibody neutralization and target for vaccine design. *Microbiol. Mol. Biol. Rev.*, **2008**, *72*(1), 54-84.

[19] Ho, J.; Uger, R.A.; Zwick, M.B.; Luscher, M.A.; Barber, B.H.; MacDonald, K.S. Conformational constraints imposed on a pan-neutralizing HIV-1 antibody epitope result in increased antigenicity but not neutralizing response. *Vaccine*, **2005**, *23*(13), 1559-1573.

[20] Joyce, J.G.; Hurni, W.M.; Bogusky, M.J.; Garsky, M.; Liang, X.; Citron, M.P.; Danzeisen, R.C.; Miller, M.D.; Shiver, J.W.; Keller, P.M. Enhancement of alpha -Helicity in the HIV-1 Inhibitory Peptide DP178 leads to an increased affinity for human monoclonal antibody 2F5 but does not elicit neutralizing responses *in vitro*. *J. Biol. Chem.*, **2002**, *277*(48), 45811-45820.

[21] Zwick, M.B.; Jensen, R.; Church, S.; Wang, M.; Stiegler, G.; Kunert, R.; Katinger, H.; Burton, D.R. Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. *J. Virol.*, **2005**, *79*(2), 1252-1261.

[22] Ofek, G.; Tang, M.; Sambor, A.; Katinger, H.; Mascola, J.R.; Wyatt, R.; Kwong, P.D. Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with its gp41 epitope. *J. Virol.*, **2005**, *78*(19), 10724-10737.

[23] Neurath, A.R.; Strick, N.; Lin, K.; Jiang, S. Multifaceted consequences of anti-gp41 monoclonal antibody 2F5 binding to HIV type 1 virions. *AIDS Res. Hum. Retroviruses*, **1995**, *11*(6), 687-696.

[24] Van Regenmortel, M.H.V. The rational design of biological complexity: a deceptive metaphor. *Proteomics*, **2007**, *7*(6), 965-975.

[25] Van Regenmortel, M.H.V. Transcending the structuralist paradigm in immunology - affinity and biological activity rather than purely structural considerations should guide the design of synthetic peptide epitopes. *Biomed. Pept. Protein Nucl. Acids*, **1995**, *1*(3), 109-116.

[26] Van Regenmortel, M.H.V.; Muller, S. *Synthetic Peptides as Antigens*; Elsevier: Amsterdam, **1999**.

[27] Saphire, E.O.; Montero, M.; Menendez, A.; van Houten, N.E.; Irving, M.B.; Pantophlet, R.; Zwick, MB.; Parren, P.W.H.I.; Burton, D.R.; Scott, J.K.; Wilson, I.A. Crystal structure of a broadly neutralizing anti-HIV-1 antibody in complex with a peptide: mechanism of gp120 cross-reactivity. *J. Mol. Biol.*, **2007**, *369*(3), 696-709.

[28] Van Regenmortel, M.H.V. Plant virus serology. *Adv. Virus Res.*, **1966**, *12*, 207-271.

[29] Van Regenmortel, M.H.V. The conformational specificity of viral epitopes. *FEMS Microbiol. Lett.*, **1992**, *100*(1-3), 483-488.

[30] Boeyé, A.; Rombaut, B. The proteins of poliovirus. *Prog. Med. Virol.*, **1992**, *62*, 139-166.

[31] Neurath, A.R.; Rubin, B.A. *Viral Structural Components as Immunogens of Prophylactic Value*. *Monographs in Virology*. Karger: Basel, **1971**.

[32] Bothner, B.; Dong, X.F.; Bibbs, L.; Johnson, J.E.; Suizdak, G. Evidence of viral capsid dynamics using limited proteolysis and mass spectrometry. *J. Biol. Chem.*, **1998**, *273*(2), 673-676.

[33] Zolla-Pazner, S. Identifying epitopes of HIV-1 that induce protective antibodies. *Nat. Rev. Immunol.*, **2004**, *4*(3), 199-210.

[34] Zwick, M.B.; Burton, D.R. HIV-1 neutralization: mechanisms and relevance to vaccine design. *Curr. HIV Res.*, **2007**, *5*(6), 608-624.

[35] Geysen, H.M.; Rodda, S.J.; Mason, T.J. A priori delineation of a peptide which mimics a discontinuous antigenic determinant. *Mol. Immunol.*, **1986**, *23*(7), 709-715.

[36] Delmastro, P.; Meola, A.; Monaci, P.; Cortese, R.; Galfre, G. Immunogenicity of filamentous phage displaying peptide mimotopes after oral administration. *Vaccine*, **1997**, *15*(11), 1276-1285.

[37] Tropsha, J; S.; Kizer, J.S.; Chaiken, I.M. Making sense from antisense: a review of experimental data and developing ideas on sense-antisense peptide recognition. *J. Mol. Recognit.*, **1992**, *5*(2), 43-54.

[38] Van Oss, C.J. Hydrophobic, hydrophilic and other interactions in epitope-paratope binding. *Mol. Immunol.*, **1995**, *32*(3), 199-211.

[39] Boquet, D.; Déry, O.; Forbert, Y.; Grassi, J.; Couraud, J.Y. Is hydropathic complementarity involved in antigen-antibody binding? *Mol. Immunol.*, **1995**, *32*(4), 303-308.

[40] Hanin, V.; Déry, O.; Boquet, D.; Sagot, M-A.; Cremoin, C.; Couraud, J.Y.; Grassi, J. Importance of hydropathic complementarity for the binding of the neuropeptide substance P to a monoclonal antibody: equilibrium and kinetic studies. *Mol. Immunol.*, **1997**, *34*(12-13), 829-838.

[41] Van Regenmortel, M.H.V. Antigenicity and immunogenicity of synthetic peptides. *Biologicals*, **2001**, *29*(3-4), 209-213.

[42] Meloen, R.H.; Puyk, W.C.; Sloostra, J.W. Mimotopes: realization of an unlikely concept. *J. Mol. Recognit.*, **2000**, *13*(6), 352-359.

[43] Larralde, OG.; Martinez, R.; Camacho, F.; Amin, N.; Aguilar, A.; Talavera, A.; Stott, D.L.; Perez, E.M. Identification of hepatitis A virus mimotopes by phage display, antigenicity and immunogenicity. *J. Virol. Methods*, **2007**, *140*(1-2), 49-58.

[44] Van Regenmortel, M.H.V. From absolute to exquisite specificity. Reflections on the fuzzy nature of species, specificity and antigenic sites. *J. Immunol. Methods*, **1998**, *216(*1-2), 37-48.

[45] James, L.C.; Roversi, P.; Tawfik, D.S. Antibody multi-specificity mediated by conformational diversity. *Science*, **2003**, *299*(5611), 1362-1367.

[46] Demchenko, A.P. Recognition between flexible protein molecules: induced and assisted folding. *J. Mol. Recognit.*, **2001**, *14*(1), 42-61.

[47] Ma, B.; Shatsky, M.; Wolfson, H.J.; Nussinov, R. Multiple diverse ligands binding at a single protein site: A matter of pre-existing populations. *Protein Sci.*, **2002**, *11*(2), 184-197.

[48] Getzoff, E.D.; Tainer, J.A.; Lerner, R.A.; Geysen, H.M. The chemistry and mechanism of antibody binding to protein antigens. *Adv. Immunol.*, **1988**, *43*, 1-98.

[49] Benjamin, D.C.; Berzofsky, J.A.; East, I.J.; Gurd, F.R.N.; Hannum, C.; Leach, S.J.; Margoliash, E.; Michael, J.G.; Miller, A.; Prager, E.M.; Reichlin, M.; Sercaz, E.E.; Smith-Gill, S.J.; Todd, PE.; Wilson, A.C. The antigenic structure of proteins: A reappraisal. *Ann. Rev. Immunol.*, **1984**, *2*, 67-101.

[50] Rubinstein, N.D.; Mayrose, I.; Halperin, D.; Yekutieli, D.; Gershoni, J.M.; Pupko, T. Computational characterization of B-cell epitopes. *Mol. Immunol.*, **2008**, *45*(12), 3477-3489.

[51] Tilley, S.A.; Honnen, W.J.; Racho, M.E.; Chou, T.C.; Pinter, A. Synergistic neutralization of HIV-1 by human monoclonal antibodies against the V3 loop and the CD4 binding site of gp120. *AIDS Res. Hum. Retroviruses*, **1992**, *8*(4), 461-467.

[52] Laal, S.; Burda, S.; Gorny, M.K.; Karwowska, S.; Buchbinder, A.; Zolla-Pazner, S. Synergistic neutralization of human immunodeficiency virus type 1 by combinations of human monoclonal antibodies. *J. Virol.*, **1994**, *68*(6), 4001-4008.

[53] Mascola, J.R.; Louder, M.K.; VanCott, T.C.; Sapan, C.V.; Lambert, J.S.; Muenz, L.R.; Bunow, B.; Birx, D.L.; Robb, M.L. Potent and synergistic neutralization of human immunodeficiency virus (HIV) type 1 primary isolates by hyperimmune anti-HIV immunoglobulin combined with monoclonal antibodies 2F5 and 2G12. *J. Virol.*, **1997**, *71*(10), 7198-7206.

[54] Zeder-Lutz, G.; Hoebeke, J.; Van Regenmortel, M.H.V. Differential recognition of epitopes present on monomeric and oligomeric forms of gp160 glycoprotein of human immunodeficiency virus type 1 by human monoclonal antibodies. *Eur. J. Biochem.*, **2001**, *268*(10), 2856-2866.

[55] Zwick, M.B.; Wang, M.; Poignard, P.; Stiegler, G.; Katinger, H.; Burton, D.R.; Parren, W.H.I. Neutralization synergy of human immunodeficiency virus type 1 primary isolates by cocktails of broadly neutralizing antibodies. *J. Virol.*, **2001**, *75*(24), 12198-12208.

[56] Poon, B.; Safrit, J.T.; McClure, H.; Kitchen, C.; Hsu, J.F.; Gudeman, V.; Petropoulos, C.; Wrin, T.; Chen, I.S.Y.; Grovit-Ferbas, K. Induction of humoral immune responses following vaccination with envelope-containing, formaldehyde-treated, thermally inactivated human immunodeficiency virus type 1. *J. Virol.*, **2005**, *79* (8), 4927-4935.

[57] Poon, B.; Hsu, J.F.; Gudeman, V.; Chen, I.S.Y.; Grovit-Ferbas, K. Formaldehyde-treated, heat-inactivated virions with increased human immunodeficiency virus type 1 env can be used to induce high-titer neutralizing antibody responses. *J. Virol.*, **2005**, *79*(16), 10210-10217.

[58] Crooks, E.T.; Moore, P.L.; Franti, M.; Cayanan, C.S.; Zhu, P.; Jiang, P.; de Vries, R.P.; Wiley, C.; Zharkikh, I.; Schülke, N.; Roux, K.H.; Montefiori, D.C.; Burton, D.R.; Binley, J.M. A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. *Virology*, **2007**, *366*(2), 245-262.

[59] Lescar, J.; Pelligrini, M.; Souchon, H.; Tello, D.; Poljak, R.J.; Peterson, N.; Greene, M.; Alzari, M. Crystal structure of a cross-reaction complex between Fab F9.13.7 and guinea fowl lysozyme. *J. Biol. Chem.*, **1995**, *270*(30), 18067-18076.

[60] Schroer, J.A.; Bender, T.; Feldmann, T.; Kim, K.J. Mapping epitopes on the insulin molecule using monoclonal antibodies. *Eur. J. Immunol.*, **1983**, *13*(9), 693-700.

[61] Edwards, B.M.; Barash, S.C.; Main, S.H.; Choi, G.H.; Minter, R.; Ullrich, S.; Williams, E.; Du Fou, L.; Wilton, J.; Albert, V.R.; Ruben, S.M.; Vaughan, T.J. The remarkable flexibility of the human antibody repertoire; isolation of over one thousand different antibodies to a single protein, BLyS. *J. Mol. Biol.*, 2003, *334*(1), 103-118.

[62] Brunel, F.M.; Zwick, M.B.; Cardoso, R.M.F.; Nelson, J.D.; Wilson, I.A.; Burton, D.R.; Dawson, P.E. Structure-function analysis of the epitope for 4E10, a humanimmunodeficiency virus type 1 (HIV-1) broadly neutralizing antibody. *J. Virol.*, **2006**, *80*(4), 1680-1687.

[63] Nelson, J.D.; Brunel, F.M.; Jensen, R.; Crooks, E.T.; Cardoso, R.M.F.; Wang, M.; Hessel, A.; Wilson, I.A.; Zwick, M.B. An affinity-enhanced neutralizing antibody against the membrane-proximal external region of human immunodeficiency virus type 1 gp41 recognizes an epitope between those of 2F5 and 4E10. *J. Virol.*, **2007**, *81*(8), 4033-4043.

[64] Javaherian, K.; Langlois, A.J.; McDanal, C.; Ross, K.L.; Eckler, L.I.; Jellis, CL.; Profy, A.T.; Rusche, J.R.; Bolognesi, D.P.; Putney, S.D.; Matthews, T.J. Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc. Natl. Acad. Sci. USA*, **1989**, *86*(17), 6768-6772.

[65] Spear, G.R.; Takefman, D.M.; Sharpe, S.; Ghassemi, M.; Zolla-Pazner, S. Antibodies to the HIV-1 V3 loop in serum from infected persons contribute a major proportion of immune effector functions including complement activation, antibody binding, and neutralization. *Virology*, **1994**, *204*(2), 609-615.

[66] VanCott, T.C.; Polonis, V.R.; Loomis, L.D.; Michael, N.L.; Nara, P.L.; Birx, D.L. Differential role of V3-specific antibodies in neutralization assays involving primary and laboratory-adapted isolates of HIV type 1. *AIDS Res. Hum. Retroviruses*, **1995**, *11*(11), 1379-1391.

[67] Beddows, S.A.; Louisirirotchanakul, S.; Cheingsong-Popov, R.; Easterbrook, P.J.; Simmonds, P.; Weber, J.N. Neutralization of primary and T-cell line adapted isolates of human immunodeficiency virus type 1: role of V3-specific antibodies. *J. Gen. Virol.*, **1998**, *79*(1), 77-82.

[68] Spenlehauer, C.; Saragosti, S.; Fleury, H.J.; Kirn, A.; Aubertin, A.M.; Moog, C. Study of the V3 loop as a target epitope for antibodies involved in the neutralization of primary isolates versus T-cell-line-adapted strains of human immunodeficiency virus type 1. *J. Virol.*, **1998**, *72*(12), 9855-9864.

[69] Kayman, S.C.; Wu, Z.; Revesz, K.; Chen, H.; Kopelman, R.; Pinter, A. Presentation of native epitopes in the V1/V2 and V3 regions of human immunodeficiency virus type 1 gp120 by fusion glycoproteins containing isolated gp120 domains. *J. Virol.*, **1994**, *68*(1), 400-410.

[70] Gorny, M.K.; Williams, C.; Volsky, B.; Revesz, K.; Cohen, S.; Polonis, V.R.; Honnen, W.J.; Kayman, S.C.; Krachmarov, C.; Pinter, A.; Zolla-Pazner, S. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. *J. Virol.*, **2002**, *76*(18), 9035-9045.

[71] Richalet-Secordel, P.; Zeder-Lutz, G.; Plaue, S.; Sommermeyer-Leroux, G.; Van Regenmortel, M.H.V. Cross-reactivity of monoclonal antibodies to a chimeric V3 peptide of HIV-1 with peptide analogues studied by biosensor technology and ELISA. *J. Immunol. Methods.*, **1994**, *176*(2), 221-234.

[72] Rini, J.M.; Schulze-Gahmen, U.; Wilson, I.A. Structural evidence for induced fit as a mechanism for antibody-antigen recognition. *Science*, **1992**, *255*(5047), 959-965.

[73] Churchill, M.E.A.; Stura, E.A.; Pinilla, C.; Appel, J.R.; Houghten, R.A.; Kono, D.H.; Balderas, R.S.; Fieser, G.G.; Schulze-Gahmen, U.; Wilson, I.A. Crystal structure of a peptide complex of anti-influenza peptide antibody Fab 26/9. Comparison of two different antibodies bound to the same peptide antigen. *J. Mol. Biol.*, **1994**, *241*(4), 534-556.

[74] Wilson, I.A.; Stanfield, R.L. Antibody-antigen interactions: new structures and new conformational changes. *Curr. Opin. Struct. Biol.*, **1994**, *4*(6), 857-867.

[75] Berger, C.; Weber-Bornhauser, S.; Eggenberger, H.; Hanes, J.; Pluckthun, A.; Bosshard, H.R. Antigen recognition by conformational selection. *FEBS Lett.*, **1999**, *450*(1-2), 149-153.

[76] Bosshard, H.R. Molecular recognition by induced fit: how fit is the concept? *News Physiol. Sci.*, **2001**, *16*(8), 171-173.

[77] Goh, C.S.; Milburn, D.; Gerstein, M. Conformational changes associated with protein-protein interactions. *Curr. Opin. Struct. Biol.*, **2004**, *14*(1), 104-109.

[78] Stanfield, R.L.; Gorny, M.K.; Zolla-Pazner, S.; Wilson, I.A. Crystal structures of human immunodeficiency virus type 1 (HIV-1) neutralizing antibody 2219 in complex with three different V3 peptides reveal a new binding mode for HIV-1 cross-reactivity. *J. Virol.*, **2006**, *80*(12), 6093-6105.

[79] Muller, S. In *Synthetic Peptides as Antigens;* Van Regenmortel, M.H.V.; Muller, S., Eds.; Elsevier: Amsterdam, **1999**, pp. 133-177.

[80] Benito, A.; Van Regenmortel, M.H.V. Biosensor characterization of antigenic site A of foot-and-mouth disease virus presented in different vector systems. *FEMS Immunol. Med. Microbiol.*, **1998**, *21*(2), 101-115.

[81] Chakraborty, K.; Durani, V.; Miranda, E.R.; Citron, M.; Liang, X.; Schleif, W.; Joyce, J.G.; Varadarajan, R. Design of immunogens that present the crown of the HIV-IV3 loop in a conformation competent to generate 447-52 D-like antibodies. *Biochem. J.*, **2006**, *399*, 483-491.

[82] Rybicki, E.P. Plant-produced vaccines: promise and reality. *Drug Discov.Today*, **2009**, *14*(1-2), 16-24.

[83] Gorny, M.K.; Xu, J-Y.; Karwowska, S.; Buchbinder, A.; Zolla-Pazner, S. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. *J. Immunol.*, **1993**, *150*(2), 635-643.

[84] Gorny, M.K.; Revesz, K.; Williams, C.; Volsky, B.; Louder, M.K.; Anyangwe, C.A.; Krachmarov, C.; Kayman, S.C.; Pinter, A.; Nadas, A.; Nyambi, P.N.; Mascola, J.R.; Zolla-Pazner, S. The V3 Loop is accessible on the surface of most human immunodeficiency virus type 1 primary isolates and serves as a neutralization epitope. *J. Virol.*, **2004**, *78*(5), 2394-2404.

[85] Rosen, O.; Chill, J.; Sharon, M.; Kessler, N.; Mester, B.; Zolla-Pazner, S.; Anglister, J. Induced fit in HIV-neutralizing antibody complexes: evidence for alternative conformations of the gp120 V3 loop and the molecular basis for broad neutralization. *Biochemistry*, **2005**, *44*(19), 7250-7258.

[86] Leinikki, P.; Lehtinen, M.; Hyoty, H.; Parkkonen, P.; Kantanen, M.L.; Hakulinen, J. Synthetic peptides as diagnostic tools in virology. *Adv. Virus Res.*, **1993**, *42*, 149-186.

[87] Gomara, M.J.; Haro, I. Synthetic peptides for the immunodiagnosis of human diseases. *Curr. Med. Chem.*, **2007**, *14*(5), 531-546.

[88] Pellequer, J.L.; Westhof, E.; Van Regenmortel, M.H.V. Predicting location of continuous epitopes in proteins from their primary structures. *Methods. Enzymol.*, **1991**, *203*, 176-201.

[89] Ponomarenko, J.V.; Van Regenmortel, M.H.V. In *Structural Bioinformatics*, 2nd., Gu, J.; Bourne, P.E., Eds.; John Wiley: Hoboken, N.J. **2009**, pp. 849-879.

[90] Pellequer, J.L.; Westhof, E.; Van Regenmortel, M.H.V. Correlation between the location of antigenic sites and the prediction of turns in proteins. *Immunol. Lett.*, **1993**, *36*(1), 83-99.

[91] Blythe, M.J.; Flower, D.R. Benchmarking B cell epitope prediction: Underperformance of existing methods. *Protein Sci.*, **2005**, *14*(1), 246-248.

[92] Greenbaum, J.A.; Andersen, P.H.; Blythe, M.; Bui, H.H.; Cachau, R.E.; Crowe, J.; Davies, M.; Kolaskar, A.S.; Lund, O.; Morrison, S.; Mumey, B.; Ofran, Y.; Pellequer, J.L.; Pinilla, C.; Ponomarenko, J.V.; Raghava, G.P.; Van Regenmortel, M.H.V.; Roggen, E.L.; Sette, A.; Schlessinger, A.; Sollner, J.; Zand, M.; Peters, B. Towards a consensus on datasets and evaluation metrics for developing B-cell epitope prediction tools. *J. Mol. Recognit.*, **2007**, *20*(2), 75-82.

[93] Hopp, T.P. Retrospective: 12 years of antigenic determinant predictions, and more. *Pept. Res.*, **1993**, *6*(4), 183-190.

[94] Van Regenmortel, M.H.V.; Pellequer, J.L. Predicting antigenic determinants in proteins: looking for unidimensional solutions to a three-dimensional problem? *Pept. Res.*, **1994**, *7*(4), 224-228.

[95] Kulkarni-Kale, U.; Bhosle, S.; Kolaskar, A.S. CEP: a conformational epitope prediction server. *Nucl. Acids Res.* **2005**, *33* (Web server issue), W168-W171.

[96] Haste A.P.; Nielsen, M.; Lund, O. Prediction of residues in discontinuous B-cell epitopes using protein 3D structures. *Protein Sci.*, **2006**, *15*(11), 2558-2567.

[97] Moreau, V.; Granier, C.; Villard, S.; Laune, D.; Molina, F. Discontinuous epitope prediction based on mimotope analysis. *Bioinformatics*, **2006**, *22*(9), 1088-1095.

[98] Moreau, V.; Fleury, C.; Piquer, D.; Nguyen, C.; Novali, N.; Villard, S.; Laune, D.; Granier, C.; Molina, F. PEPOP: computational design of immunogenic peptides. *BMC Bioinform.*, **2008**, *9*, 71.

[99] Bublil, E.M.; Freund, N.T.; Mayrose, I.; Penn, O.; Roitburd-Berman, A.; Rubinstein, N.D.; Pupko, T.; Gershoni, J.M. Stepwise prediction of conformational discontinuous B-cell epitopes using the Mapitope algorithm. *Proteins*, **2007**, *68*(1), 294-304.

[100] Sweredoski, M.J.; Baldi, P. PEPITO: improved discontinuous B-cell epitope prediction using multiple distance thresholds and half sphere exposure. *Bioinformatics*, **2008**, *24*(12), 1459-1460.

[101] Oomen, C.J.; Hoogerhout, P.; Bonvin, A.M.; Kuipers, B.; Brugghe, H.; Timmermans, H.; Haseley, S.R.; van Alphen, L.; Gros, P. Immunogenicity of peptide-vaccine candidates predicted by molecular dynamics simulations. *J. Mol. Biol.*, **2003**, *328*(5), 1083-1089.

[102] Enshell-Seijffers, D.; Denisov, D.; Groisman, B.; Smelyanski, L.; Meyuhas, R.; Gross, G.; Denisova, G.; Gershoni, J.M. The mapping and reconstitution of a conformational discontinuous B-cell epitope of HIV-1. *J. Mol. Biol.*, **2003**, *334*(1), 87-101.

[103] Villen, J.; de Oliveira, E.; Nunez, J.I.; Molina, N.; Sobrino, F.F.; Andreu, D. Towards a multi-site synthetic vaccine to foot-and-mouth disease: addition of discontinuous site peptide mimic increases the neutralization response in immunized animals. *Vaccine*, **2004**, *22*(27-28), 3523-3529.

[104] Dakappagari, N.K.; Lute, K.D.; Rawale, S.; Steele, J.T.; Allen, S.D.; Phillips, G.; Reilly, R.T.; Kaumaya, P.T. Conformational HER-2/*neu* B-cell epitope peptide vaccine designed to incorporate two native disulfide bonds enhances tumor cell binding and antitumor activities. *J. Biol. Chem.*, **2005**, *280*(1), 54-63.

[105] Misumi, S.; Nakayama, D.; Kusaba, M.; Iiboshi, T.; Mukai, R.; Tachibana, K.; Nakasone, T.; Umeda, M.; Shibata, H.; Endo, M.; Takamune, N.; Shoji, S. Effects of immunization with CCR5-Based cycloimmunogen on Simian/HIVSF162P3 challenge. *J. Immunol.*, **2006**, *176*(1), 463-471.

[106] Timmerman, P.; Beld, J.; Puijk, W.C.; Meloen, R.H. Rapid and quantitative cyclization of multiple peptide loops onto synthetic scaffolds for structural mimicry of protein surfaces. *Chembiochem*, **2005**, *6(5)*, 821-824.

[107] Timmerman, P.; Puijk, W.C.; Meloen, R.H. Functional reconstruction and synthetic mimicry of a conformational epitope using CLIPS technology. *J Mol. Recognit.*, **2007**, *20*(5), 283-299.

[108] DeLano, W.L. Unraveling hot spots in binding interfaces: progress and challenges. *Curr. Opin. Struct. Biol.*, **2002**, *12*(1), 14-20.

[109] Van Regenmortel, M.H.V. Reductionism and the search for structure-function relationships in antibody molecules. *J. Mol. Recognit.*, **2002**, *15*(5), 240-247.

[110] Van Regenmortel, M.H.V. Reductionism and complexity in molecular biology. *EMBO Reports*, **2004**, *5*(11), 1016-1020.

Received: December 13, 2008 Revised: January 28, 2009 Accepted: March 11, 2009